REMARKS

Claims 1-22 are pending in this application. Claims 9, 10, 12 and 13 have been cancelled. Claims 1, 3, 4, 6, 11 and 14 have been amended to overcome the rejection of these claims under 35 USC 112, second paragraph.

The specification has been objected to and accordingly amended, as shown above and in response to the Examiner's request, to update the status of the parent application recited in the specification amendment filed on January 26, 2004. No new matter has been added. Withdrawal of this objection is accordingly requested.

The present specification is objected to under 37 CFR 1.821(d) because the Examiner finds the nucleotide sequences and the protein sequence n Figures 1 and 6-7 should be identified with SEQ ID NOs:, respectively. Accordingly, the brief descriptions of those figures on pages 6-9 of the present specification have been amended to recited the identifiers, as suggested by the Examiner. No new matter has been added. Withdrawal of this objection is accordingly requested.

Claims 1, 4, 5, 7, 8, 11 and 14 stand rejected under 35 USC 112, second paragraph because the Examiner finds claim 1 to be unclear by the recitation of "both said DNA fragment and expression of a promoter" and unclear as to the other element of "both." Claim 1 a has accordingly been amended in view of the Examiner's remarks to a clearer form, as shown above.

Additionally, the Examiner finds the recitation of "of a peptide-coding sequence" to be unclear and suggests inserting the language of "in a plant cell or a plant" into the claims. Claims 3, 4, 6, 11 and 14 have accordingly been so amended as shown above.

The applicants respectfully submit that all pending claims are fully allowable under Section 112, second paragraph.

The applicants respectfully traverse the rejection of claims 1-8, 11 and 14 under 35 USC 112, first paragraph for the following reasons.

The Office Action states that the present specification does not enable any person of ordinary skill in the art to make or use the presently claimed invention commensurate in scope with claims 1, 4, 5, 7, 8, 11 and 14.

In particular, the Office Action states that there is no evidence that SEQ ID NO: 2 alone could drive light repressible expression without combining with other promoters or other parts of SEQ ID NO: 3.

The applicants respectfully disagree. The present specification discloses that a 12-bp cis-element (SEQ ID NO: 2) consisting of the 12-bp core sequence alone is sufficient to confer light repressibility on the expression of a gene placed downstream of the element. The applicants point out that Example 9 describes an analysis which was made to determine whether or not the 12-bp cis-element has the ability to confer light responsiveness on the minimal promoter of CaMV 35S (CaMV 35S46) comprising the -46 bp region. The applicants submit that claim 5 satisfies the enablement requirement.

Further, the Office Action states that there is no evidence to suggest that SEQ ID NO: 1 alone (one copy) or any sequence containing SEQ ID NO: 1 could be functional as SEQ ID NO: 2, 3, or the sequence containing nine copies of SEQ ID NO: 1.

The applicants respectfully traverse this contention and attach as documentary support, the technical reference, Mitsuhara et al., Plant Cell Physiol.

37(1) pages 49-59 (1996). This reference describes that a series of chimeric promoters for higher-level expression of foreign genes in plants was constructed as fusions of CaMV 35S transcript (Fig. 1 of reference), the strength of these promoters was assayed in expression systems in plants and the activity of the promoter was evaluated. Mitsuhara et al. describes that the each chimeric promoter, which had different numbers of tandem repeats of cis-element from the 5'-upstream region of the 35S promoter, respectively, had promoter activity (Table 1).

Further, Mitsuhara also describes that cis-element had activity such as an activity which enhances gene expression, autofluorescence activity. This meant that the expression was inhibited by light and so on regardless of its repeating number. Based upon the teachings of the Mitsuhara reference, the applicants submit that a person of ordinary skill in the art would understand that there is evidence to suggest that both SEQ ID NO: 1 alone (one copy) and any sequence containing SEQ ID NO: 1 could be functional as the sequence containing nine copies of SEQ ID NO: 1. Therefore, the skilled artisan can easily carry out the present invention on the basis of the technical acknowledge at the filing date of the present application, such as that described in the Mitsuhara reference.

Additionally, the Office Action states that there is no evidence suggesting that the elements such as SEQ ID NO: 1 to 3 would likely work similarly in plants other than pea plants. The applicants respectfully disagree. The reference of Ngai et al., cited in the Office Action, Form PTO 892, Notice of References Cited, (Ngai, et al., The Plant Journal (1997), 12(5), pages 1021-1034) describes that cis-element affecting the expression of asparagines synthetase (AS) genes whose transcription is negatively regulated by light and the cis-element were found in nuclear extracts of

tobacco, pea and Arabidopsis and therefore be universal factors involved in light-activated transcriptional repression. Further, Ngai, et al. also describes that the identity of "repressor" elements in AS1 would be useful for engineering a promoter for temporal expression of foreign genes in plants in which foreign gene expression could be repressed by light and/or sucrose (see page 1032 left column lines 9 to 13). From the standpoint of the Ngai et al. reference, the applicants submit that a person of ordinary skill in the art would understand that there is evidence suggesting that the elements which relate to the light-repressed transcription would likely work similarly in plants other than pea.

Accordingly, the applicants submit that all presently considered claims are fully allowable under Section 112, first paragraph.

The applicants respectfully traverse the rejection of claims 1-3 and 7 under 35 USC 102(b) in view of Jansen et al. or Okubo et al. or Richards et al.

None of the cited references anticipate the presently claimed invention or make it obvious.

The DNA fragment containing the nucleic sequence of SEQ ID NO: 1 of the presently claimed invention is clearly differentiated from the nucleic acid disclosed in Jansen et al., Okubo et al., and Richards et al. in chemical structure, and especially the length of the nucleic sequence. The nucleic sequence of SEQ ID NO: 1 of the presently claimed invention is not identical as a substance to the sequences disclosed in those references because the length of the SEQ ID NO: 1 is different from the length of the referenced sequences.

Importantly, the references Jansen et al., Okubo et al. or Richards et al. neither disclose nor teach that the nucleic sequence of SEQ ID NO: 1 of the presently claimed invention is a core sequence.

Moreover, the references Jansen et al., Okubo et al. or Richards et al. neither disclose nor teach that "an isolated DNA fragment is characterized by expressions of both a peptide-coding sequence placed downstream of the DNA fragment and a promoter operatively linked to said peptide-coding sequence in a plant cell or a plant are repressed by irradiation with white light at 70 μmole/m^2/sec or irradiation with red light for 2 minutes".

Accordingly, the applicants submit that the presently claimed invention is not only unanticipated under Section 102 (b) by the references Jansen et al., Okubo et al. or Richards et al., but further, the presently claimed invention is not rendered obvious by the teachings of these references taken alone or in combination. The presently claimed invention is fully allowable in view of the cited prior art.

The applicants respectfully traverse the rejection of claims 4-6 and 8 under 35 USC 102(b) or in the alternative, under 35 USC 103(a) in view of Richards et al. The applicants submit that this reference does not make the presently claimed invention to be obvious.

The promoter containing the nucleic sequence of SEQ ID NO: 1 of the presently claimed invention is clearly differentiated from the nucleic acid disclosed in Richards et al. in chemical structure, especially the length of the nucleic sequence as discussed above. The nucleic sequence of SEQ ID NO: 1 of the presently claimed invention is not identical as a substance to the sequences disclosed in Richards et al. because the length of the SEQ ID NO: 1 is different from the length of the

reference sequences. Further, Richards, neither discloses nor suggests that the nucleic sequence of SEQ ID NO: 1 of the presently claimed invention is a core sequence upstream of the promoter.

Importantly, Richards et al. neither discloses nor suggests that the promoter is characterized by expression of a peptide-coding sequence operatively linked downstream of the promoter in a plant cell or a plant is repressed by irradiation with white light at 70 μmole/m^2/sec or irradiation with red light for 2 minutes.

The applicants submit that the presently claimed invention is no where disclosed, suggested or made obvious by the cited art. The presently claimed invention is fully allowable under both Section 102(b) and Section 103(a) in view of the cited references.

In view of the above and the attached two technical references, the applicants submit that this application is in condition for allowance and a Notice to that effect is respectfully requested.

Respectfully submitted,

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TECHNICAL REFERENCES

1) Mitsuhara et al., Plant Cell Physiol. 37(1) pages 49-59 (1996)

2) Ngai, et al., The Plant Journal (1997), 12(5), pages 1021-1034

Plant Cell Physiol. 37(1): 49-59 (1996)

Efficient Promoter Cassettes for Enhanced Expression of Foreign Genes in Dicotyledonous and Monocotyledonous Plants

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A series of chimeric promoters for higher-level expression of foreign genes in plants was constructed as fusions of a gene for β-glucuronidase (GUS) with the terminator of a gene for nopaline synthase (*nos*) or of the cauliflower mosaic virus (CaMV) 35S transcript, and the strength of these promoters was assayed in transient and stable expression systems in tobacco and rice. As parts of these promoters, the CaMV 35S core promoter, three different 5′-upstream sequences of the 35S promoter, the first intron of a gene for phaseolin, and a 5′-untranslated sequence (Ω sequence) of tobacco mosaic virus were used in various combinations. In tobacco and rice protoplasts, all three fragments of the 35S promoter (−419 to −90, −390 to −90 and −290 to −90, relative to the site of initiation of transcription), the intron, and the Ω sequence effectively enhanced GUS activity. Some chimeric promoters allowed levels of GUS activity that were 20- to 70-fold higher than those obtained with the 35S promoter in pBI221. In tobacco protoplasts, the two longer fragments of the 35S promoter were more effective than the shortest fragment. In rice cells, by contrast, the shortest fragment was as effective as the two longer ones. The terminator of the 35S transcript was more effective than that of the *nos* gene for gene expression. In transgenic tobacco plants, a representative powerful promoter, as compared to the 35S promoter, allowed 10- and 50-fold higher levels of expression on average and at most, respectively, with no clear qualitative differences in tissue- and organ-specific patterns of expression. When the representative promoter was introduced into tobacco with a gene for luciferase, the autofluorescence of detached leaves after a supply of luciferin to petioles was great and was easily detectable by the naked eye in a dark room.

Key words: CaMV 35S promoter — Gene expression — Rice (*Oryza sativa*) — Strong promoter — TMV Ω sequence — Tobacco (*Nicotiana tabacum*).

Promoters regulate gene expression both quantitatively and qualitatively. The regulatory sequences of promoters that define the qualitative specificity of gene expression in plants have been studied and much information has accumulated in recent years (Hennig et al. 1994). However, details of the fine regulation of levels of the expression of transgenes have not been easy to obtain because of the limited kinds of promoter available. The cauliflower mosaic virus (CaMV) 35S promoter, which has been most frequently used as a constitutive strong promoter in plants (Benfey and Chua 1990, Terada and Shimamoto 1990, Yang and Christou 1990) results in a level of expression that is insufficient for some purposes. For example, content of coat protein was only 0.1% of the total leaf protein and resistance to infection by tobacco mosaic virus (TMV) was not very great in a transgenic tobacco plant that contained a chimeric gene composed of the 35S promoter and the gene for the TMV coat protein (Powell et al. 1986). Because the level of viral resistance is dependent on the level of accumulated coat protein (Beachy et al. 1990), resistance should be improved if the level of expression of the coat protein gene could be increased by use of a promoter stronger than the 35S promoter. The level of expression of an introduced antisense gene driven by the 35S promoter was also found to be insufficient in transgenic plants for complete inhibition of expression of ADP-glucose pyrophosphorylase (Müller-Röber et al. 1992) and a sucrose transporter (Reismeier et al. 1994).

Abbreviations: CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyl transferase; GUS, β-glucuronidase; LUC, luciferase; 4-MU, 4-methyl-umbelliferyl glucuronide; *nos*, gene for nopaline synthase gene, PCR, polymerase chain reaction; TMV, tobacco mosaic virus; X-gluc, 5-bromo-4-chloro-3-indolyl glucuronide.

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Several other sequences have been reported to enhance the expression of foreign genes. A 5'-upstream region of the 35S promoter (−343 to −90) was found to act as an enhancer sequence in higher plants (Odell et al. 1988). It contains many types of *cis* element that confer tissue-specific expression (Fang et al. 1989, Benfey and Chua 1990). When this upstream region was used in two tandem repeats as an enhancer, the level of gene expression increased with increases in the number of regions (Kay et al. 1987, Timmermans et al. 1990, Omirulleh et al. 1993).

Introns in some genes have been postulated to stimulate gene expression by increasing the stability of the corresponding mRNAs in the case of both animal cells and their viruses (Gruss and Khoury 1980, Buchman and Berg 1988, Huang and Gorman 1990). Certain plant introns have also been shown to increase the level of expression of homologous or heterologous genes (Callis et al. 1987, McElroy et al. 1990, Clancy et al. 1994). When the first intron of a gene for phaseolin, a storage protein of the common bean *Phaseolus vulgaris* (Slightom et al. 1983), was inserted in the 5'-upstream region of a gene for chloramphenicol acetyl transferase (CAT), a 10-fold enhancement of gene expression was observed in rice protoplasts (Hirochika et al. in preparation).

Tobacco mosaic virus (TMV) has a unique G-free sequence (Ω sequence) in the 5'-untranslated region of its genomic RNA. This sequence was reported to improve the efficiency of translation in plant, animal and *E. coli* cells, in vitro and in vivo (Sleat et al. 1987, Gallie et al. 1991), probably by preventing the formation of secondary structure of mRNA and/or enhancing interactions between mRNA and ribosomes.

The terminator of chimeric genes also plays an important role in gene expression by affecting the stability of mRNA (Ingelbrecht et al. 1989). In plants, the 3'-untranslated region of the CaMV 35S transcript and that of the gene for nopaline synthase (*nos*) in the Ti plasmid (Bevan et al. 1983) are often used as terminators.

Although the individual elements listed above have been reported to affect gene expression, there have been no reports on the effects of combinations of these sequences together in monocotyledonous and dicotyledonous plants. In this study, we constructed a series of promoter cassettes that contained specific sequences in an attempt to induce higher levels of expression of transgenes in plants. The levels of expression of the various constructs were analyzed to evaluate the effects of the different elements in transient and stable expression systems in tobacco and rice.

Materials and Methods

Construction of promoter-GUS chimeric genes—DNA was manipulated as described by Maniatis et al. (1989), and recombinant polymerase chain reaction (PCR) was carried out as described by Higuchi (1989). Plasmids pBI221 and pBI121 (Jefferson et al. 1987) were products of Clontech (Palo Alto, CA, U.S.A.). Plasmid pFF19G (Timmermans et al. 1990) was a gift from Prof. J. Messing (Rutgers University). Plant expression vectors pREX-1, pEN4 and pEN6 were made by Hirochika et al. (in preparation). The gene for luciferase (LUC) was obtained as a restriction fragment from pT3/T7-Luc (Clontech).

The promoter region of the CaMV 35S transcript was amplified by PCR from pBI221 with A5 (5'-ATCTCCACTGACGTAAGGGATGACG-3') and A3 (5'-TTGTAAAAATACGTACCTCTCCAAATGAAATGAACTTCC-3') as primers. The Ω sequence of TMV was amplified from TMV cDNA clone pL_{11} A-A25 (Nishiguchi et al. 1985) with B5 (5'-TTTCATTTGGAGAGGTACGTATTTTTACAACAATTACCAACAA-3') and B3 (5'-GTACGAGCTCTGATCAACGTCCATGGTGGATCCTCTAGATGTAGTTGTAGAATGTAAAATGTAATGTTG-3') as primers.

Closed circular plasmid DNA for electroporation was purified by CsCl/ethidium bromide density gradient centrifugation. The GUS fusion in the binary vector pBI121 (Clontech) was replaced by constructed GUS fusions, inserted at the *Hin*dIII and *Eco*RI sites. Each resulting construction was used to transform *Agrobacterium tumefaciens* strain LBA4404 (Ooms et al. 1981) by electroporation.

Analysis of transient expression—The transient expression of GUS fusion constructs was analyzed as follows. Tobacco mesophyll protoplasts were prepared from fully expanded upper leaves of *Nicotiana tabacum* cv. Samsun NN. The lower epidermis of a sterilized tobacco leaf was abraded with carborundum (mesh 600; Kishida Chemicals Co., Osaka, Japan) to facilitate penetration of the wall-degrading enzymes, and the pieces of leaves were floated on 0.5 M mannitol that contained 1% cellulase Onozuka R-10 (Yakult Co., Tokyo, Japan) and 0.02% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) at 28°C with occasional agitation. After digestion for 2 h, the protoplasts were filtered through 80-μm nylon mesh, collected by centrifugation at 100 × *g* for 2 min and then washed twice with 0.5 M mannitol. The viability of the protoplasts was determined by staining with Evans Blue (Wako Chemicals Co., Osaka, Japan).

Isolated protoplasts (10^5 cells) were carefully resuspended in 0.5 ml of 0.5 M mannitol that contained 10 μg of purified supercoiled plasmid DNA which included various promoter fusion constructs and 100 μg of carrier DNA (salmon sperm DNA, 0.5 kb in average length; Wako Chemicals Co.). Electroporation was performed at 500 V cm^{-1} for 15–20 ms on ice, in a cuvette with a 0.4-cm electrode gap, in a dielectrophoretic cell-fusion processor (model SCF1A; Sankei Co., Tokyo, Japan). Electroporated protoplasts were kept on ice for 15–30 min, collected by centrifugation, and then cultured at 28°C in darkness in 0.5 ml of medium that contained inorganic salts (Murashige and Skoog 1962), vitamins for B5 medium (Gamborg et al. 1968), 5 μM 2,4-D, 2.5 μM kinetin and 0.4 M glucose.

Rice protoplasts were prepared from suspension-cultured cells of *Oryza sativa* cv. Nipponbare. Isolation and electroporation were carried out as described previously (Kosugi et al. 1990).

Analysis of stable expression—Tobacco plants were transformed by infection with *A. tumefaciens* LBA4404 that carried the modified binary vectors by the leaf disc-method (Horsch et al. 1985). Transformants were selected in the presence of 100 μg ml^{-1} kanamycin. GUS activity in the upper fully-expanded leaves of regenerated transformants was analyzed at a similar growth stage.

Transformation of rice was carried out as described by Sugimoto et al. (1994). Protoplasts were prepared from rice sus-

pension-cultured cells (cv. Nipponbare). Cells (1.5×10^6 ml^{-1}) were electroporated in a continuous flow electro-transfector (CET-100; JASCO, Tokyo, Japan) in 0.5 M mannitol that contained 0.1 mM $MgSO_4$, 20 μg ml^{-1} sonicated calf thymus DNA as carrier DNA, 2 μg of plasmid DNA that included a fusion construct and 0.4 μg of plasmid that contained the CaMV 35S promoter and the gene for hygromycin phosphotransferase (pUC19HPT; kindly provided by Dr. A. Kato) as a selectable marker gene. A rectangular electric pulse of 1,000 V cm^{-1} was applied six times for 0.1 ms each. After a 10-day incubation in a conditioned medium, hygromycin-resistant calli were selected and integration of the fusion constructs was studied by PCR.

Analysis of GUS and LUC activities—GUS activity was analyzed by the fluorometric method of Kosugi et al. (1990) with 4-methyl-umbelliferyl glucuronide (4-MU) as a substrate. Histochemical staining for GUS was carried out as described by Ohshima et al. (1990). Tissue or pollen was stained in 50 mM phosphate buffer (pH 7.0) that contained 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) at 37°C in the presence of antibiotics. For the assay of LUC activity, expanded upper young leaves of transgenic plants that contained the LUC gene were detached. After absorption of 200 μl of a 1 mM solution of luciferin (Sigma, St. Louis, MO, U.S.A.) in 10 mM sodium-citrate buffer (pH 5.0) through the petiole, the leaves were photographed in a dark room on ISO 1600 film, with an exposure time of 10 min, and an aperture of F/1.7.

Results

Construction of promoter cassettes—The structures of the promoter cassettes that were constructed in this study are summarized in Figure 1. The cassettes were generated as follows. The CaMV 35S core promoter was prepared by PCR using primers A5 and A3. A3 includes a *Sna*BI site and a sequence complementary to the 5′ region of the Ω sequence of TMV at its 5′ end. The Ω sequence was prepared by PCR with primers B5 and B3. The B5 primer has a sequence identical to the 3′ end of the CaMV 35S promoter, and the B3 primer has a polylinker sequence that contains *Xba*I, *Bam*HI, *Nco*I, *Bcl*I and *Sac*I sites. Products of PCR with the 35S promoter and the Ω sequence were fused by the recombinant PCR technique.

The *Nco*I site of the plant expression plasmid pREX-1, located in the 5′-upstream region of the 35S enhancer, was disrupted by digestion with *Nco*I that was followed by filling in with the Klenow fragment and self-ligation. The *Eco*RV-*Sac*I region of the resultant plasmid pREX-1′ was

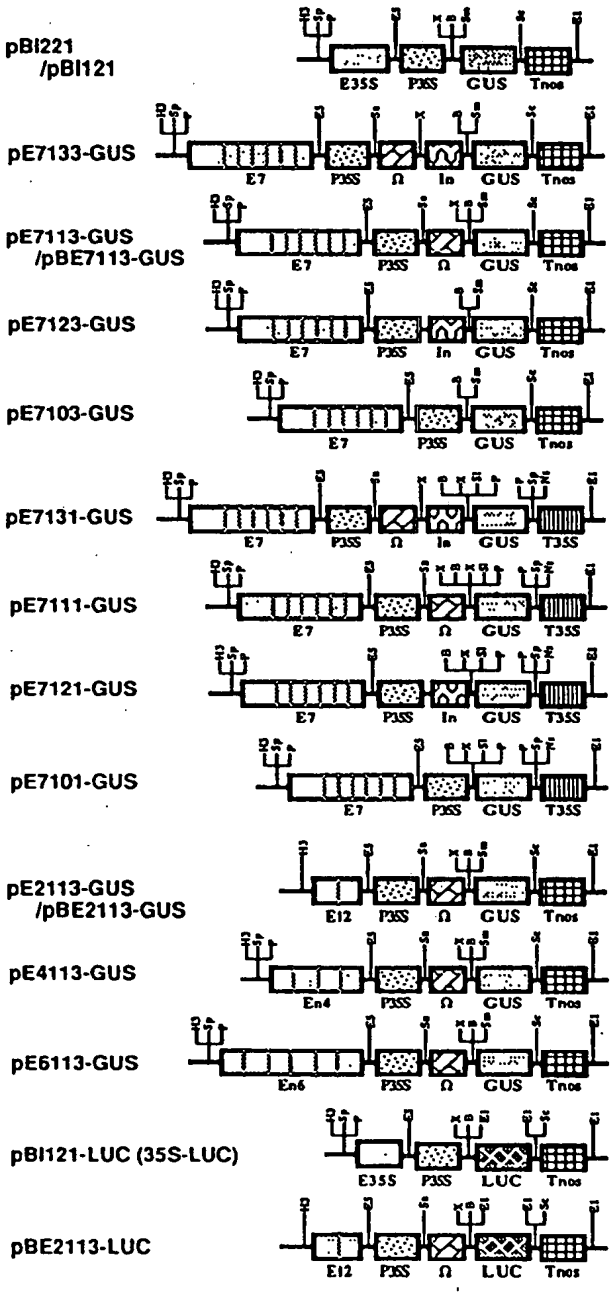

Fig. 1 Diagrams of all tested constructs. All constructs were inserted into *Hind*III and *Eco*RI restriction sites located within the polycloning sites of plasmid pUC18 or pBI121. E7: 5′-upstream sequence of CaMV 35S promoter (−940 to −290) and (−290 to −90) × 7. EI2: 5′-upstream sequence of CaMV 35S promoter (−419 to −90) × 2. En4: 5′-upstream sequence of CaMV 35S promoter (−940 to −390) and (−390 to −90) × 4. En6: 5′-upstream sequence of CaMV 35S promoter (−940 to −390) and (−390 to −90) × 6. P35S: 5′-upstream sequence of CaMV 35S promoter (−90 to −1). Ω: 5′-untranslated sequence of TMV. Intron: first intron of a gene for phaseolin. GUS: protein-coding region of a gene for β-glucuronidase. LUC: protein-coding region of a gene for luciferase. Tnos: polyadenylation signal of the gene for nopaline synthase in the Ti plasmid. T35S: polyadenylation signal of the CaMV 35S transcript. B, *Bam*HI; E1, *Eco*RI; E5, *Eco*RV; H3, *Hind*III; Ns, *Nsi*I; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; Sn, *Sna*BI; Sp, *Sph*I; P, *Pst*I; X, *Xba*I.

replaced by a product of recombinant PCR that contained the 35S core promoter and the Ω sequence to make pE7113 (E7Ω). The plasmid pE7113 has seven tandemly repeated enhancer-like elements (−90 to −290, relative to the site of initiation of transcription as +1) of the 35S promoter (E7), the 35S core promoter, the Ω sequence, the polylinker sequence, and the terminator of the *nos* gene. The first intron of the gene for phaseolin was obtained as an *Xba*I-*Bam*HI fragment of pREX-1 and inserted into the *Xba*I-*Bam*HI site of pE7113 (E7Ω) to make pE7133 (E7ΩIn). Each regulatory sequence utilized in pE7133 (E7ΩIn) has unique flanking restriction sites those allow the sequences to be easily removed or replaced by other sequences. Accordingly, pE7123 (E7In) and pE7103 (E7) were produced by excision of the Ω sequence from pE7133 and pE7113, respectively, by *Sna*BI and *Xba*I digestion, blunting and ligation. The GUS gene was obtained as a *Bam*HI-*Sac*I fragment from pBI221 and inserted into pE7133, pE7113, pE7123 and pE7103 to produce pE7133-GUS (E7ΩIn-GUS), pE7113-GUS (E7Ω-GUS), pE7123-GUS (E7In-GUS) and pE7103-GUS (E7-GUS), respectively.

Replacement of the *nos* terminator of the plasmid by the 35S terminator and cloning of the GUS gene was achieved by cloning the *Bam*HI-*Eco*RI fragment of pFF19G into the *Bam*HI and *Eco*RI sites in pE7133, pE7113, pE7123 and pE7103 to produce pE7131-GUS, pE7111-GUS, pE7121-GUS and pE7101-GUS, respectively. The enhancer-like element of pE7113 was replaced by *Hin*dIII and *Eco*RV fragments of pFF19G, pEN4, or pEN6. The resulting clones had different lengths and copy numbers of enhancer-like elements from the 5'-upstream region of the 35S promoter; pE2113 had two tandem repeats of −419 to −90 (El2), pE4113 had four tandem repeats of −390 to −90 (En4), and pE6113 had six repeats of −390 to −90 (En6). The *Hin*dIII and *Eco*RI fragments were excised from pE7113-GUS (E7Ω-GUS) and pE2113-GUS (El2Ω-GUS) and recloned into a binary Ti plasmid pBI121 to yield the binary plasmids pBE7113-GUS (E7Ω-GUS) and pBE2113-GUS (El2Ω-GUS), respectively. In order to produce the expression vectors that contained the gene for LUC (pBI121-LUC and pBE2113-LUC), the gene for LUC was obtained as a *Bam*HI-*Sac*I fragment from pT3/T7-Luc and recloned into binary plasmids pBI121 (35S-GUS) and pBE2113-GUS (El2Ω-GUS), respectively, at the *Bam*HI and *Sac*I site.

Analysis of transient expression in tobacco and rice protoplasts—To evaluate the promoter activities of the various constructs, the level of GUS activity in tobacco and rice protoplasts was examined one day after electroporation (Table 1). In tobacco mesophyll protoplasts, the level of GUS activity was about two or more orders of magnitude higher than that in rice protoplasts prepared from suspension-cultured cells on the basis of both cell number and protein concentration. All promoters presented in Table 1 conferred considerably higher GUS activity than the 35S promoter of pBI221 (35S-GUS). As compared to the level of expression of 35S-GUS, the levels of expression of tested constructs were 4- to 26-fold higher in tobacco cells and 1.5- to 76-fold higher in rice cells.

The Ω sequence of TMV increased GUS activity 2- to

Table 1 Transient expression of the GUS gene driven by various promoter constructs in tobacco and rice protoplasts

Name of plasmid	Structure of plasmid	GUS activity (4-MU nmol/10^7 cells/min)			
		Tobacco	(Ratio)	Rice	(Ratio)
pBI221	35S - GUS - Tnos	50	(1.0)	0.27	(1.0)
pE7133-GUS	E7 - P35S - Ω - In - GUS - Tnos	860	(17.2)	18.5	(68.5)
pE7113-GUS	E7 - P35S - Ω - GUS - Tnos	430	(8.6)	5.6	(20.7)
pE7123-GUS	E7 - P35S - In - GUS - Tnos	195	(3.9)	1.9	(6.9)
pE7103-GUS	E7 - P35S - GUS - Tnos	Not tested		0.40	(1.5)
pE7131-GUS	E7 - P35S - Ω - In - GUS - T35S	1,170	(23.4)	20.5	(75.9)
pE7111-GUS	E7 - P35S - Ω - GUS - T35S	1,320	(26.4)	7.5	(27.8)
pE7121-GUS	E7 - P35S - In - GUS - T35S	725	(14.5)	4.4	(16.3)
pE7101-GUS	E7 - P35S - GUS - T35S	305	(6.1)	1.8	(6.5)
pE2113-GUS	El2 - P35S - Ω - GUS - Tnos	1,150	(23.0)	0.96	(3.6)
pE4113-GUS	En4 - P35S - Ω - GUS - Tnos	860	(17.2)	2.4	(9.0)
pE6113-GUS	En6 - P35S - Ω - GUS - Tnos	1,300	(26.0)	3.2	(11.9)

After electroporation, protoplasts were incubated for 24 h, and GUS activity was determined as described in Materials and Methods. The values relative to those obtained with pBI221 (35S-GUS construct) are shown in parentheses. All data are averages of results from four different experiments.

5-fold in tobacco and 4- to 10-fold in rice, as revealed by comparison of the activities of promoter constructs with and without Ω (pE7133-GUS vs. pE7123-GUS, pE7113-GUS vs. pE7103-GUS, pE7131-GUS vs. pE7121-GUS and pE7111-GUS vs. pE7101-GUS). In all four cases, the Ω sequence was quite effective in enhancing gene expression. Thus, this sequence was effective in both tobacco and rice cells.

The positive effect of the intron on the expression of fusion constructs was also clear in rice protoplasts. Comparison of data for pE7133-GUS vs. pE7113-GUS and pE7121-GUS vs. pE7101-GUS revealed an intron-mediated 3- to 5-fold enhancement of GUS activity. In tobacco cells, the effect of the intron was less clear, it induced only a doubling of GUS activity when pE7133-GUS (E7ΩIn-GUS) was compared to pE7113-GUS (E7Ω-GUS) and pE7121-GUS to pE7101-GUS. Moreover, the intron had no effect on pE7131 as compared to pE7111.

All enhancer-like sequences of the 35S promoter used in this study [El2, (−419 to −90)×2; En4, (−940 to −390)+(390 to −90)×4; En6, (−940 to −390)+(−390 to −90)×6; and E7, (−940 to −290)+(−290 to −90)×7] increased GUS activity in both tobacco and rice cells but the extent of such enhancement varied. In tobacco cells, the two longer fragments with fewer repeats (El2, En4 and En6) conferred higher-level expression than the shorter sequence with more repeats (E7). For example, the levels of the activity associated with pE2113-GUS (El2Ω-GUS), pE4113-GUS (En4Ω-GUS) and pE6113-GUS (En6Ω-GUS) were 2 to 3 times higher than that associated with pE7113-GUS (E7Ω-GUS). By contrast, in rice cells, the shortest enhancer with the most repeats (E7) was the most effective sequence, being 2 to 6 times more active than the longer enhancers with fewer repeats (En4, En6 and El2), as indicated by the transient expression of GUS from pE7113-GUS (E7Ω-GUS), pE4113-GUS (En4Ω-GUS), pE6113-GUS (En6Ω-GUS) and pE2113-GUS (El2Ω-GUS).

The effect of the terminator in the fusion constructs was studied with four different sets of promoters, namely, pE7133-GUS and pE7131-GUS, pE7113-GUS and pE7111-GUS, pE7123-GUS and pE7121-GUS, and pE7103-GUS and pE7101-GUS. In all cases, the terminator of the CaMV 35S transcript induced higher levels of GUS activity than that of the *nos* gene in both plant species (Table 1).

In tobacco protoplasts, pE2113 (El2Ω), pE6113 (En6Ω), pE7131 and pE7111 similarly caused extremely high-level expression equal to more than 20 times that of 35S-GUS. Among these constructs, pE2113 (El2Ω) was selected as the representative of promoters of highest-level expression in dicotyledonous plants for further analysis because it was the shortest and had the simplest structure. The pE7113 construct (E7Ω), with an expression level that was 8.6 times that of 35S-GUS, was selected as a moderately high-level expression promoter.

In rice protoplasts, pE7133 (E7ΩInTnos) and pE7131 (E7ΩInT35S) induced exceptionally high levels of expression, which were about 70 times higher than that induced by 35S-GUS. Construct pE7133 (E7ΩInTnos) was selected as a representative of the highest-level expression promoters in monocotyledonous plants because of the ease of its construction, while pE7113 (E7Ω) was taken as a moderately high-level expression promoter, causing expression at a level 21-fold higher than 35S-GUS.

Analysis of the promoters in tobacco and rice transfor-

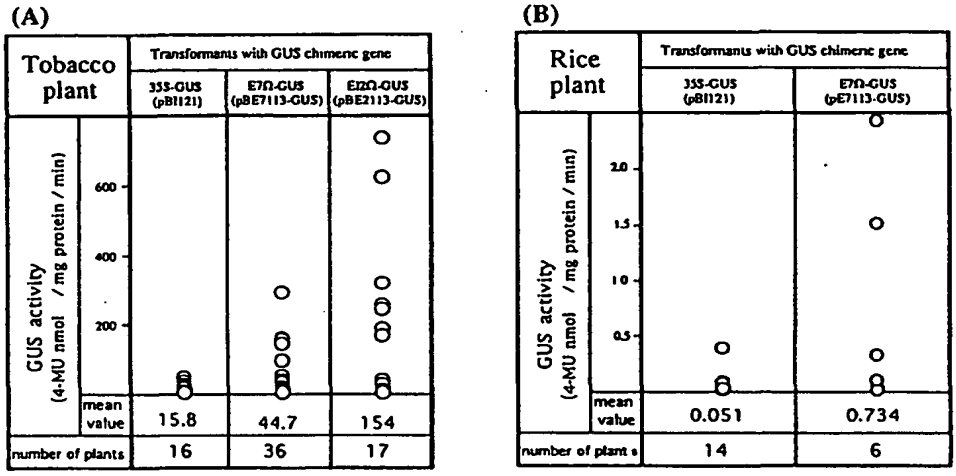

Fig. 2 Frequency distribution of levels of GUS activity in transgenic tobacco (A) and rice plants (B). Mean GUS activities and the numbers of plants used are shown at the bottom of the Figure.

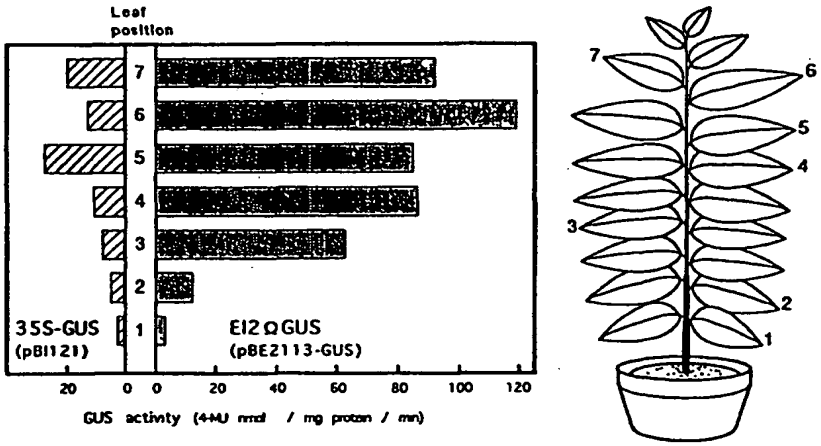

Fig. 3 Effect of leaf position on GUS activity in transgenic tobacco plants that carried 35S-GUS (pBI121) and EI2Ω-GUS (pBE2113-GUS). Data were obtained from a typical specimen of each transgenic plant.

mants—To study the characteristics of the constructed promoters in transgenic tobacco plants, *Hin*dIII and *Eco*RI fragments containing the entire promoter-GUS-terminator constructs from pE7113-GUS (E7Ω-GUS) and pE2113-GUS (EI2Ω-GUS) were inserted into the *Hin*dIII-*Eco*RI site of the binary vector pBI121, replacing of 35S-GUS gene. The resultant plasmids were introduced into *Agrobacterium tumefaciens* LBA4404. After inoculation of pieces of tobacco leaves with the bacterium, 30 to 40 kanamycin-resistant shoots were regenerated and rooted. The regenerated plants were transferred to soil, and GUS activity in the upper fully developed leaves was measured. No major differences were observed in the efficiency of transformation and regeneration or in morphology among all 30–40 transformants and the control non-transformants. As shown in Figure 2A, transformants with the EI2Ω-GUS and E7Ω-GUS promoters had apparently higher GUS activity than those with the 35S promoter. Compared with the level in transformants that carried 35S-GUS, the level of GUS activity in E7Ω-GUS and EI2Ω-GUS transformants was about 3 and 10 times higher on average, respectively. The highest levels of GUS activity in EI2Ω-GUS plants, E7Ω-GUS plants, and 35S-GUS plants were 733, 289, and 45 nmoles 4-MU mg protein^{-1} min^{-1}, respectively.

GUS activity in the middle parts of leaf blades of young transgenic rice plants that harbored pE7113-GUS (E7Ω-GUS) and pBI221 (35S-GUS) was determined. The frequency distribution of GUS activities in leaf extracts of transgenic rice is shown in Figure 2B. The overall level of GUS activity was much higher in the E7Ω-GUS population, being on average about 14 times higher than that in 35S-GUS plants. The highest levels of GUS activity in transformants with E7Ω-GUS and in those with 35S-GUS were 2.4 and 0.37 nmoles 4-MU mg protein^{-1} min^{-1}, respectively.

It is important to characterize the specific mode expression of promoters at each developmental and spatial level. GUS activities of leaves at different positions on 35S-GUS and EI2Ω-GUS tobacco plants at a similar growth stage (when plants were about 120 cm in height) were determined (Fig. 3). In both 35S-GUS and EI2Ω-GUS plants, the activity was highest in the upper fully expanded leaves (leaf position 5–7 in Fig. 3), decreasing gradually in the leaves at lower position.

Histochemical analysis of the expression of strong promoters in transgenic tobacco plants—To study the mode of gene expression of the strong promoters, GUS activity was determined by histochemical staining of organs and tissues in transgenic tobacco plants. Figure 4 shows GUS activities in cross sections of leaf blades (A, B), longitudinal sections

Fig. 4 Histochemical examination of the expression of foreign genes in transgenic tobacco plants. Several parts of tissues or organs from transgenic tobacco (A–L) were subjected to analysis of GUS activity. (A, C, E, G, I, K) Transgenic tobacco plants containing pBI121 (35S-GUS). (B, D, F, H, J, L) Transgenic tobacco plants containing pBE2113-GUS (EI2Ω-GUS). (A, B) Leaf sections (100 μm thick) stained for 2 h at 37°C. Bar = 200 μm. (C, D) Longitudinal sections of shoot apex (80 μm thick) stained for 1 h. Bar = 500 μm. (E, F) Longitudinal sections of flowers, 4 days before flowering, stained for 1 h. Bar = 500 μm. (G, H) Intact pollen grains stained for 2 h. Bar = 20 μm. (I, J) Intact roots stained for 1 h. Bar = 100 μm. (K, L) Intact seedlings, 4 days after imbibition, stained for 1 h. Bar = 500 μm. (M) Autofluorescence of transgenic tobacco leaves that contained pBI121-LUC (left) and pBE2113-LUC (right). Luciferin was absorbed by leaves of transformants from petioles and autofluorescence was observed in a dark room. The photograph was taken on ISO 1600 film, with an exposure time of 10 min, and an aperture of F/1.7.

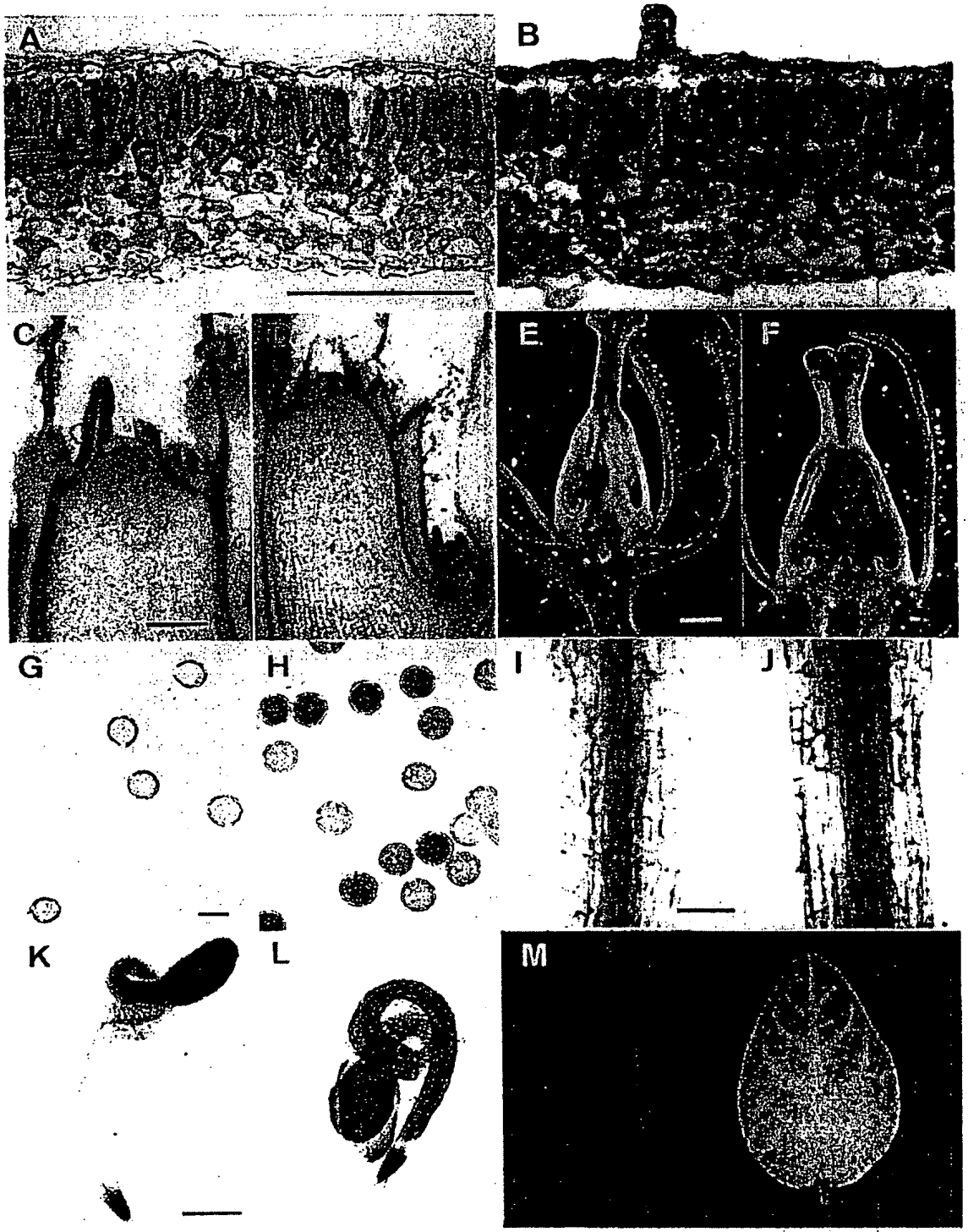
A
B
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Table 2 GUS activity in various organs of transgenic tobacco seedlings of R1 progeny that harbored 35S-GUS and El2Ω-GUS

Organ	35S-GUS			El2ΩGUS		
	Stage of germination			Stage of germination		
	1[a]	2[b]	3[c]	1	2	3
Cotyledon	++	++	+	++	++	+
Hypocotyl	++	−	+−	++	−	−
Transitional region between shoot and root	+	+	+−	++	++	+
Lateral root primordium	/	/	+−	/	/	+−
Stele of seminal root	/	+	+	/	++	+
Epidermis and root hair of seminal root	±	+−	+−	++	++	++
Root apical meristem	±	±	++	+	+	++
Root cap	+	−	+−	+	++	++

[a] Stage 1: seed coat has broken and radicle is extending. Days after imbibition (DAI) 2–3.
[b] Stage 2: radicle is elongating and cotyledons are extending from seed coat. (DAI 4).
[c] Stage 3: seed coat is dropping off and hypocotyl is extending from the transitional region between the shoot and the root (DAI 5–7).
+−, GUS activity was detected in some plants; ±, faint GUS activity was observed; +, GUS activity was observed; ++, strong GUS activity was observed; −, no activity was detected; /, unable to observe the organ in this stage.

of the shoot apex (C, D) and flowers (E, F) 4 days before flowering, in pollen (G, H), in roots (I, J) and in seedlings (K, L) of transgenic tobacco plants that contained 35S-GUS (pBI121; A, C, E, G, I, K) and El2Ω-GUS (pE2113-GUS; B, D, F, H, J, L). The levels of GUS activity in plants with El2Ω-GUS were much higher than those in plants with 35S-GUS in all organs and tissues examined. This result is consistent with the results obtained by fluorometric determinations of GUS activity in transient and stable expression assays (Table 1 and Fig. 2).

In leaf blades of 35S-GUS and El2Ω-GUS plants, strong GUS activity was detected in the palisade, spongy and epidermal cells (A, B). In longitudinal sections of shoot apices, strong staining was observed around vascular bundles in both 35S-GUS plants and El2Ω-GUS plants (C, D), and blue staining was also detected around apical meristems in El2Ω-GUS plants. In flower sections, GUS activity was observed only in some parts of the pistil and placenta of pBI121 plants, while strong GUS activity was observed in most parts of flowers of El2Ω-GUS plants (E, F). Pollen of El2Ω-GUS plants expressed high-level GUS activity, but pollen of 35S-GUS plants did not (G, H). While strong staining was observed in the central cylinder of roots in both types of plant, the level of GUS activity in El2Ω-GUS plants was much higher than that in 35S-GUS plants (I, J). In seedlings of 35S-GUS plants, weak activity was observed in the transition regions between shoots and roots, and between the root apical meristem and the root cap (K). In El2Ω-GUS plants, GUS activity was quite strong in roots and cotyledons (L).

The pattern of expression of GUS activity in tobacco seedlings at various developmental stages was also analyzed [Table 2 and Fig. 4 (K, L)]. In most organs, staining for GUS was stronger in tissues of El2Ω-GUS plants than in those of 35S-GUS plants. However, as shown in Table 2, the patterns of staining in the various organs were very similar in the 35S-GUS plants and El2Ω-GUS plants.

To further evaluate the promoter activities, the gene for LUC was used instead of the gene for GUS. Fusions with the LUC gene (pBE2113-LUC and pBI121-LUC, see Fig. 1) were introduced into tobacco plants. The leaves of transformants of 30 cm in height were detached and luciferin was absorbed through petioles. Among 17 transformants that carried a construct with a strong promoter (pBE2113; El2Ω), seven plants exhibited strong autofluorescence and another seven plants fluoresced weakly. The light emitted by the leaves was easily detected by the naked eye in a dark room. In the case of 35S-LUC plants, only four out of eleven transformants showed faint autofluorescence. Typical 35S-LUC and El2Ω-LUC plants that exhibited the strongest autofluorescence are contrasted in Figure 4 (M).

Discussion

We constructed a series of promoter cassettes by combining enhancers of the 35S promoter, an intron, and the Ω sequence to stimulate the expression of foreign genes in rice and tobacco. Studies of transient and stable expression demonstrated that all the cassettes could function efficiently in both tobacco and rice plants. Compared with the CaMV 35S promoter in pBI221, the strongest promoters were 26 and 76 times stronger in terms of the enhancement of levels of the marker protein in tobacco and rice protoplasts, respectively. The most efficient promoters in tobacco cells were not the same as those in rice cells, suggesting differences in the specificity of gene expression between

plant species and/or between dicotyledonous and monocotyledonous plants.

The CaMV 35S promoter is known to function in many organs of many higher plants (Benfey and Chua 1990, Terada et al. 1990, Yang and Christou 1990). Enhancer-like elements are located in the 5'-upstream region of the promoter (Odell et al. 1988, Fang et al. 1989), and tandem duplication of sequences enhances gene expression (Kay et al. 1987, Timmermans et al. 1990, Omirulleh et al. 1993). Hirochika et al. (in preparation) showed that one sequence (−290 to −90) of the 35S promoter was more effective than a longer sequence (−390 to −90) that included the shorter sequence in inducing high-level expression of CAT gene fusions in rice cells. CAT activity increased with the number of each sequence, reaching a maximum with seven tandem repeats. In the current study, seven repeats of the shorter 200-bp fragment were more effective than four and six repeats of the longer sequence (−390 to −90, En) and two repeats of the longer sequence (−419 to −90, El2) in rice cells. In tobacco cells, however, the seven repeats of the shorter 200-bp sequence were not as effective as the other two longer sequences (Table 1). This variable response to the enhancer sequences in different plant species suggests that differences exist in the transcriptional machinery, which includes specific *trans*-acting factors in individual organs. It is known that the 35S promoter has multiple *cis* elements in its 5'-upstream region (Kawata et al. 1989, Lam and Chua 1989, Yanagisawa and Izui 1992). Combinations of these elements may have different effects in different tissues, organs and plant species (Fang et al. 1989, Benfey and Chua 1990).

Some introns are known to increase the level of gene expression in animals and plants. This phenomenon is presumed to be due to an increase in the stability of mRNA, although this phenomenon is not clear in dicot plants (McElroy et al. 1991, Leon et al. 1991), perhaps because of differences in splicing efficiency between monocotyledonous and dicotyledonous plants (Keith and Chua 1986, Tanaka et al. 1990). In this study, higher GUS activity was clearly evident when we included the first intron of the gene for phaseolin in rice and sometimes in tobacco, confirming that the intron effectively enhanced the synthesis of products of a foreign gene, at least in rice plants.

The terminator in chimeric genes also plays an important role in the control of the level of gene expression by affecting the efficiency of the 3'-processing and/or the stability of mRNA (Ingelbrecht et al. 1989). In this study, it was revealed that 3'-untranslated region of the CaMV 35S transcript was more effective for higher-level expression than the terminator of the gene for nopaline synthase in the Ti plasmid. This observation indicates that selection of the terminator is also important for expression of a foreign gene.

The Ω sequence of TMV can increase the amount of a gene product in plant and animal cells, and even in *E. coli* (Sleat et al. 1987, Gallie et al. 1989, 1991). In the present study, constructs containing this sequence yielded 2 to 4 times higher GUS activity in tobacco and 3 to 5 times higher GUS activity in rice than constructs without this sequence. Because the positive effect of the Ω sequence is exerted at the translational level, the mechanism of action of this element in enhanced gene expression is probably independent of that of enhancers or introns.

The elements tested in this study might function at different steps in gene expression. Thus, all the elements, when combined, should enhance gene expression in an additive manner. Our current results that all elements enhanced expression of a reporter gene in every combination, with the exception of one case of the intron in tobacco cells (pE7131-GUS vs. pE7111-GUS), strongly support such postulate.

Among the four promoter constructs which were extraordinarily active (more than 20 times more active than 35S-GUS) in tobacco protoplasts, one construct, El2Ω, was selected together with the moderately strong promoter E7Ω (8.6 times more active) for stable transformation experiments. The levels of GUS activity in El2Ω-GUS plants and E7Ω-GUS plants were 10-fold and 3-fold higher than those in 35S-GUS plants, respectively, suggesting that the activity of a promoter in transient assays reflects its activity in transgenic plants. Histochemical analysis showed that tissue- and organ-specific expression of these promoters was very similar to that of 35S-GUS.

In the experiments with rice protoplasts, we selected two promoter constructs, pE7133 and pE7113, for generation of stable transformants. The former is a representative construct with seven repetitions of the shortest enhancer, the Ω sequence, and the intron, and it was associated with the highest level of expression (about 70 times higher than that of 35S-GUS). Transgenic calli that harbored pE7133-GUS and pE7133-LUC had the highest levels of GUS and LUC activities (data not shown), but the calli were not easy to maintain and it was hard to regenerate whole plants from them. It is possible that the negative result reflects some physiological stress caused by too large an amount of the foreign gene product. We obtained transgenic rice plants that harbored E7Ω-GUS with moderately high-level expression in rice protoplasts. On average, the GUS activity obtained with E7Ω-GUS was 14 times higher than that obtained with 35S-GUS. Although E7Ω was not the strongest expression promoter in rice protoplasts, the level of expression of the GUS gene in stable transformants that harbored E7Ω-GUS was one order higher than that with 35S-GUS.

For convenient comparisons, the effect of each element in stimulating promoter activity in tobacco and rice is summarized in Table 3.

The copy number of integrated genes might also affect

Table 3 Strength of each element as part of the promoter in the regulation of gene expression

	Element in promoter					Terminator	
	E7	En4 or En6	El2	In	Ω	Tnos	T35S
Tobacco	+	++	++	±	+	+	++
Rice	++	+	+	++	+	+	++

±, stimulatory effect was observed in some constructs; +, stimulatory effect was observed in all constructs; ++, strong stimulatory effect was observed in all constructs.

the level of expression in transgenic plants. In this study, in about two-thirds of the self-pollinated progeny of each El2Ω-GUS transgenic tobacco plant, the transgene was introduced at a single locus, as judged by the segregation of resistance to kanamycin. This fraction is similar to that for 35S-GUS plants.

The CaMV 35S promoter has often been used as a strong and constitutive promoter of the expression of many foreign genes for the production of transgenic plants in many plant species. Indeed, many experiments have shown that the 35S promoter is useful as a strong promoter for the expression of sense and antisense genes. However, in some cases, stronger promoters have been required to achieve higher levels of gene products. The powerful promoter cassettes reported here were 20- to 80-fold and 10-fold stronger than the 35S promoter in protoplasts and in transgenic plants, respectively. Using the powerful promoter El2Ω, we have obtained many kinds of useful transgenic plant, for example, virus-resistant plants with a gene for the coat protein of a potyvirus (bean yellow mosaic virus; Nakamura et al. 1994) and a antisense RNA gene of cucumber mosaic virus with and without ribozyme sequences (Nakamura et al. 1995). Another example is a transgenic plant with high-level production of asparagine (Kan et al., in preparation). Thus, these promoters are potentially useful for the production of at least 10-fold higher amounts of foreign gene products in transgenic plants both for basic studies of problems in plant molecular biology and for practical purposes. We can see the improved efficiency of a promoter for gene expression in Figure 4, in particular in the photograph of the autofluorescence of the transgenic tobacco plant with the strong promoter (Fig. 4M). Unique restriction sites located at the ends of each part of the promoters allow convenient replacement of any part of the chimeric genes by other sequences. Therefore, our effective promoter cassettes should also be useful for the analysis of the effects of given sequences on gene expression and also for the development of even more effective new cassettes.

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Light-induced transcriptional repression of the pea *AS1* gene: identification of *cis*-elements and transfactors

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Summary

Here, we examine the cis-elements and trans-factors affecting the expression of *asparagine synthetase* (*AS*) genes whose transcription is negatively regulated by light. The promoters for the *AS1* and *AS2* genes of pea were isolated, sequenced, and functionally dissected for their ability to confer regulated expression to the *GUS* reporter gene in transgenic tobacco. Histochemical analysis of transgenic plants demonstrated that the AS1 and AS2 promoters show identical patterns of cell-specific expression. The more highly active AS1 promoter was further demonstrated to confer negative light-regulation to the *GUS* gene in transgenic tobacco. Deletion analysis and gain-of-function experiments showed that 124 bp of the AS1 promoter was sufficient to confer light-activated repression to a heterologous promoter. Potential conserved transcription regulatory elements, Box B, Box C, and Box C′ within this region were shown to bind to nuclear proteins by gel shift analysis. A light-specific DNA:protein interaction was detected with Box B. The nuclear factors that bind to Box C and C′ elements of AS1 are competed by a putative repressor element 'RE1' defined previously in the oat phytochrome gene whose transcription is also repressed by light. The Box B and C/C′-Box/RE1-binding factors were found in nuclear extracts of tobacco, pea, and *Arabidopsis* and may therefore be universal factors involved in light-activated transcriptional repression.

Introduction

In plants, glutamine-dependent asparagine synthetase catalyzes the synthesis of asparagine from aspartate and glutamine. Asparagine serves to store assimilated nitrogen as well as to transport nitrogen from sources to sinks during plant development (Sieciechowicz *et al.*, 1988). Biochemical, physiological and molecular studies suggest that asparagine synthesis in plants is a dynamic process that is tightly regulated throughout development and by environmental factors such as light (Lam *et al.*, 1994; Sieciechowicz *et al.*, 1988; Tsai and Coruzzi, 1990,1991). Early studies showed that the amino acid asparagine is preferentially synthesized in the dark (Urquhart and Joy, 1981). The physiological significance of this is that asparagine, having a higher N:C ratio, is a more economical nitrogen-transport compound compared to glutamine and is used to transport nitrogen under carbon-limiting conditions (e.g. in the dark) (Lea and Miflin, 1980). Consistent with these studies is the finding that asparagine synthetase (AS) activity is higher in extracts of peas grown in the dark versus light-grown plants (Joy *et al.*, 1983).

More recent studies on *AS* gene expression extend the above cited physiological and biochemical findings to the molecular level. Two cDNA clones encoding glutamine-dependent *AS* (*AS1* and *AS2*) were originally isolated from pea using a heterologous cDNA for human *AS* as a molecular probe (Tsai and Coruzzi, 1990). Molecular studies revealed that both *AS1* and *AS2* genes are expressed at high levels in dark-grown or dark-adapted plants, and that light represses their transcription (Tsai and Coruzzi, 1991). The repression of *AS* gene transcription by light in pea and *Arabidopsis* was shown to be mediated at least in part by phytochrome (Tsai and Coruzzi, 1990; Lam *et al.*, 1994). More recent studies indicate that light may also act indirectly to mediate changes in *AS* gene expression via light-induced changes in carbon metabolism. In particular, it has been shown that sucrose supplementation can mimic the negative effects of light by repressing *AS* gene expression in dark-adapted *Arabidopsis* (Lam *et al.*, 1994). Similarly, it has been shown that the level of AS mRNA increases with the decline of sucrose content in post-harvested asparagus spears (Davis and King, 1993). These molecular studies regarding sucrose repression of *AS* gene expression reflect previous physiological studies which showed that sugar has a negative effect on the levels of the AS enzyme (Brouquisse *et al.*,1992; Genix *et al.*, 1994; Stulen and Oaks, 1977). In maize root tips, AS activity was shown to increase with sugar starvation (Brouquisse *et al.*, 1992). By contrast, AS activity decreases in maize roots when plants are treated with exogenous glucose (Stulen and Oaks, 1977).

Aside from AS, there are only a few other cases where light has been shown to regulate plant gene expression in a negative manner, examples include genes encoding for phytochrome (Bruce *et al.*, 1989; Colbert *et al.*, 1983; Lissemore and Quail, 1988; Sharrock and Quail, 1989), protochlorophyllide reductase (Mosinger *et al.*, 1985), HMG

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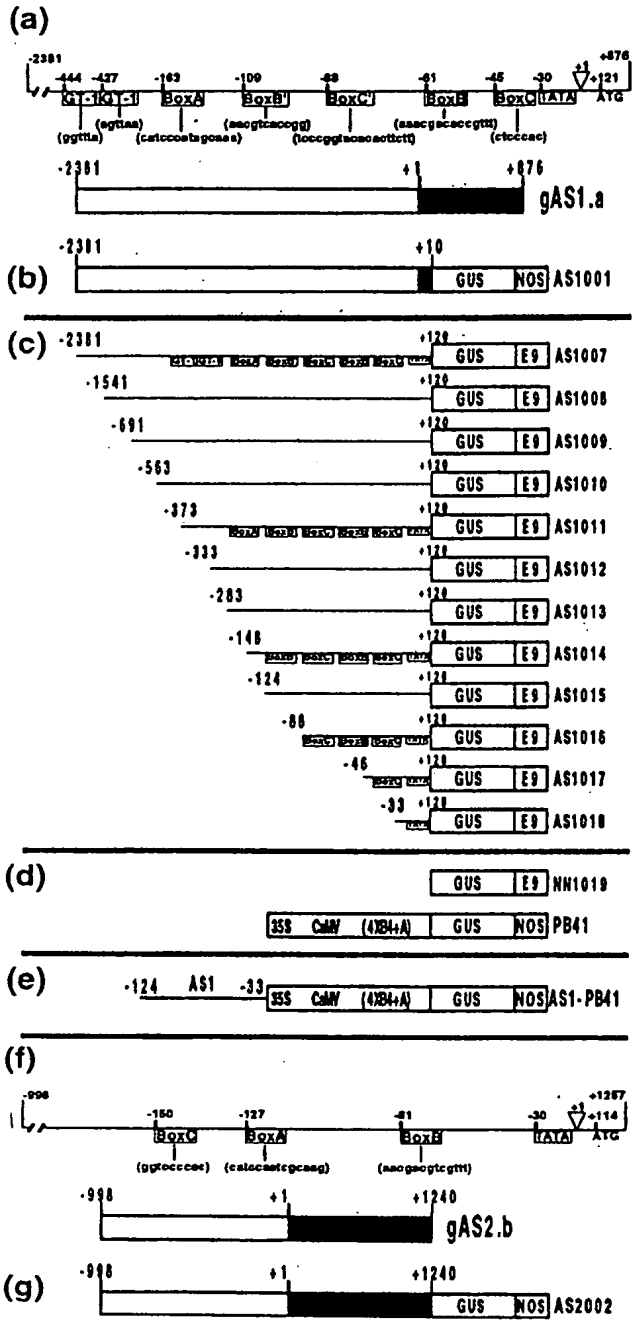
CoA reductase (Learned, 1996), and the putative transcription factor Athb-2 (Carabelli *et al.*, 1996). The molecular mechanisms by which light-activated transcriptional repression occurs is not yet understood.

Here, a functional analysis of the AS1 and AS2 promoters was performed to begin to determine the cis-acting DNA elements that effect light-repressed transcription. We demonstrate that the AS1 promoter is able to confer negative light-regulation to a reporter gene in transgenic tobacco seedlings. Deletion analysis and a gain-of-function experiment demonstrated that 124 bp of the AS1 promoter was sufficient to confer light-activated transcriptional repression to a heterologous promoter. By gel-shift and sequence analysis, potential transcription regulatory elements Box B, Box C, and Box C' were identified within this region. Two of these elements (Box B and Box C) are conserved within *AS1*, *AS2* and *pea phytochrome*, genes that are all negatively regulated by light (Sato, 1988; Tsai and Coruzzi, 1991). A light-specific factor that binds to Box B was detected. Factor binding to Boxes C and C' is competed by a putative repressor element 'RE1' previously identified in the oat phytochrome promoter (Bruce *et al.*, 1991). Therefore, Box C and Box C' are postulated to be repressor binding DNA elements involved in light-repressed transcription of the *AS1* gene. These DNA elements (Box B and Box C/C') may be used to effect the temporal expression of a foreign gene (in the dark) in transgenic plants.

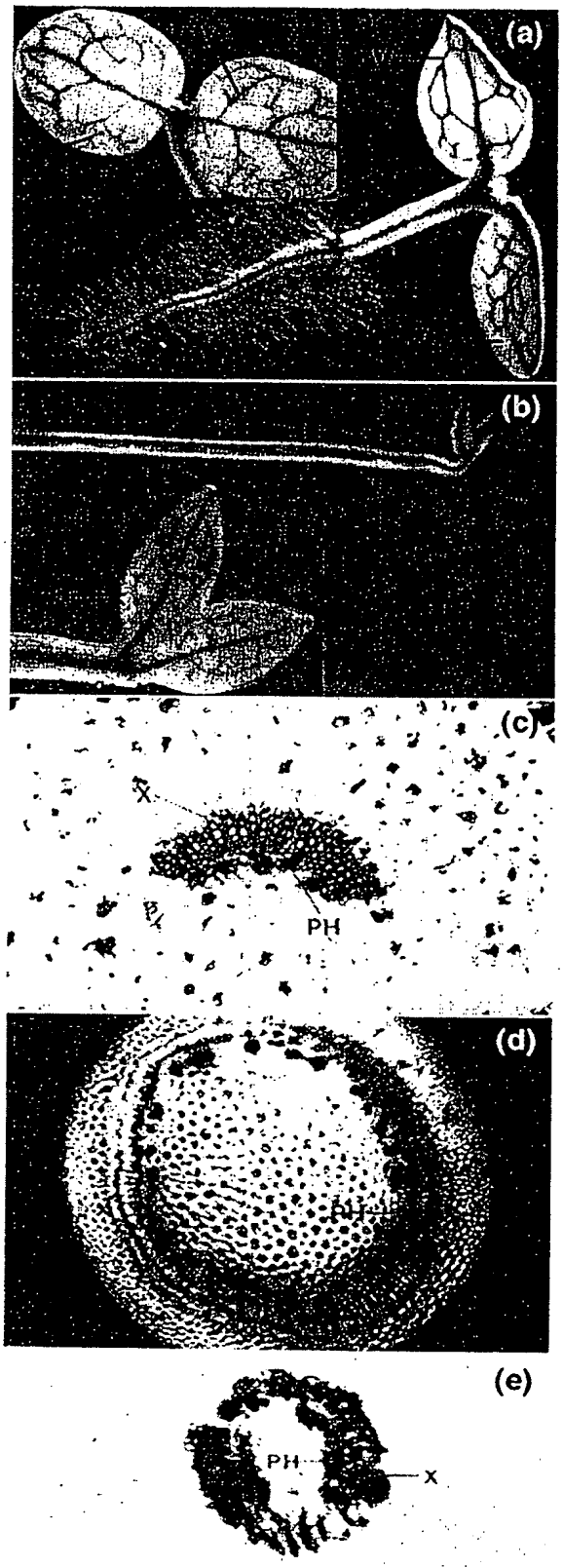


Results

Defining the promoter sequence and transcriptional initiation sites of the AS1 *and* AS2 *genes*

The AS1 and AS2 promoter regions were analysed to begin to dissect the mechanism of light-activated transcriptional repression. Figure 1(a) shows a schematic representation (not drawn to scale) of the gAS1.a fragment containing the *AS1* gene from −2381 to +876. Figure 1(f) shows that of the gAS2.b fragment containing the *AS2* gene from −998

Figure 1. AS1–GUS and AS2-GUS fusion constructs for transgenic tobacco plants.
(a) A schematic representation (not drawn to scale) of the gAS1.a fragment containing the *AS1* gene from nucleotide −2381 to +876 (+1 represents the transcription initiation site, indicated by ▽). The initiation ATG codon is indicated. GT-1 DNA binding sites (Tsai and Coruzzi, unpublished data), cis-elements identified by sequence homology conserved between AS1 (from −563 to +10), AS2 (from −998 to −1), and pea phytochrome (from −908 to −1) (Box A, B', B, and C), Box C' and the 'TATA' are boxed in the AS1 and/or AS2 promoter (see (f) below).
(b) Construct AS1001 has an AS1 fragment from −2381 to +10 in a transcriptional fusion with the GUS reporter gene.
(c) 5' AS1 deletion constructs contain the 5' AS1 untranslated leader to +120 fused to a GUS coding sequence that has the consensus sequence for eukaryotic translation flanking the ATG, and a 3' non-coding region from the rbcs-E9 gene. Cis-elements are indicated.
(d) NN1019 is a promoterless–GUS construct. The control construct, PB41 has four copies of the B4 subdomain of CaMV(−301 to −208) plus the A subdomain(−90 to +8) fused to GUS and was generated as described in Benfey *et al.* (1990).
(e) AS1–PB41 has the AS1 promoter from −124 to −33 placed upstream to the PB41 element.
(f) A schematic representation (not drawn to scale) of the gAS2.b fragment containing the *AS2* gene from nucleotide −998 to +1257.
(g) Construct AS2002 contains an AS2 fragment from −998 to +1257 in a translational fusion with the GUS reporter gene.



to +1257 (not drawn to scale). The EMBL accession number for the AS1 promoter sequence is Y13321 and for the AS2 promoter sequence, Y13322. The transcriptional initiation sites of the AS1 and AS2 genes were determined by RNase T2 assay (data not shown) and are numbered as +1 (Figure 1a and 1f). A putative TATA box for both *AS1* and *AS2* gene (TATAAAT) was found at nucleotide −30 (Figure 1a and 1f). In the RNase T2 protection assay, the AS1 was expressed at higher levels than AS2 in the RNA samples isolated from various organs, including leaves, stems and roots of dark-grown plants (data not shown). Protected AS1 and AS2 RNA products were also detected at high levels in cotyledons of germinating seedlings and in nodules (data not shown).

The AS1 and AS2 promoters confer qualitatively identical cell-specific expression patterns to the GUS reporter gene

To determine whether the AS1 and the AS2 promoters confer similar or distinct cell- or organ-specific expression patterns, AS1–GUS and AS2-GUS fusion constructs were introduced into tobacco (Figure 1b and 1g). For AS1, the promoter-GUS construct AS1001 contains −2381 to +10 of gAS1.a subcloned in a transcriptional fusion with the GUS (β-glucuronidase) reporter gene. For AS2, construct AS2002 contains nucleotides −998 to +1257 of gAS2.b subcloned in a translational fusion with the GUS reporter gene. T_1 seeds collected from the Kan^R primary transformants (T_0) were germinated on MSK medium in continuous light or in continuous darkness (etiolated). Seedlings of at least 8–10 independent transgenic tobacco lines of AS1001 and AS2002 were analysed. While each promoter showed qualitatively the same GUS expression patterns, the AS1 promoter containing construct AS1001 showed significantly higher activity compared to AS2. The average GUS activity for AS1001 light-grown 10-day old seedlings was 0.310 nmol 4-MU min^{-1} mg^{-1} (n=10 independent transformants) and for AS2002, it was 0.130 nmol 4-MU min^{-1} mg^{-1} (n=8 independent transformants). The increased activity of the AS1 promoter in transgenic plants is consistent with the finding that AS1 mRNA accumulates

Figure 2. Histochemical localization of GUS activity conferred by the AS1 promoter in transgenic tobacco plants.
GUS assays were performed on AS1001 (−2381 bp) whole mounts of (a) 10-day-old light-grown and (b) 10-day-old dark-grown transgenic tobacco seedlings. GUS assays were also performed on cross sections of leaves (c), stems (d), and roots (e) made from a representative AS1010 (−563 bp) transgenic tobacco plant grown to maturity (60 days) and dark-adapted for 2 days. Transgenic plants AS1001, AS1007–1016, and AS2002 all had identical GUS expression patterns as shown in the figure. Blue staining indicates GUS positive cells. C, cotyledon; RT, root tip; V, vasculature. PH, phloem; X, xylem.

to a fivefold higher steady state level compared to AS2 mRNA in peas (Tsai and Coruzzi, 1990).

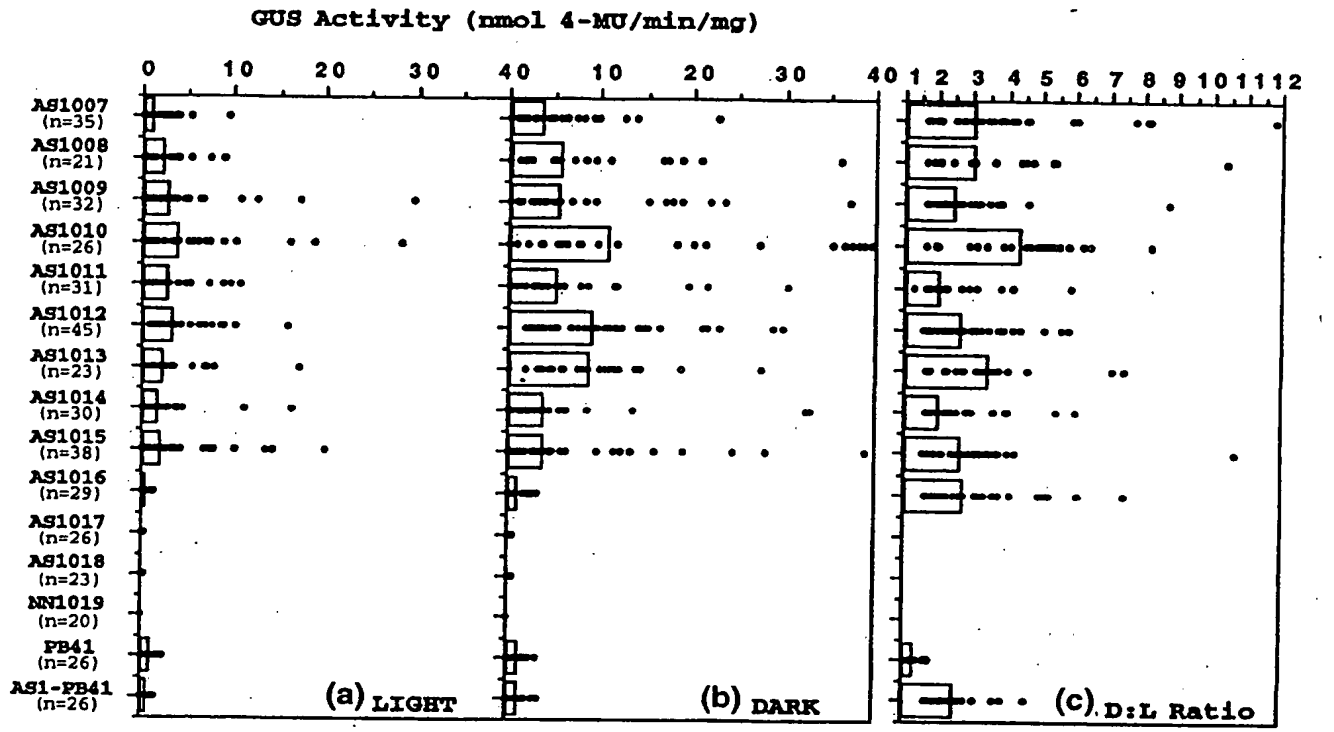
The cell-specific pattern of expression was identical in all the AS1001 and AS2002 transgenic tobacco seedlings. Germinating T_1 light- and dark-grown plants were assayed for GUS expression (Figure 2). In the initial stages of germination (days 2–4), GUS expression is detected in the endosperm and in all cells of the cotyledon (day 6) in light- or dark-grown seedlings (data not shown). By day 10, GUS expression is restricted to the vasculature in light-grown (Figure 2a) and dark-grown (Figure 2b) seedlings. In plants grown to maturity, the *AS1–GUS* gene is expressed exclusively in the vascular tissue, specifically in the internal phloem of leaves, stems, and roots of dark-adapted plants (Figure 2 c–e, respectively). In light-grown plants, GUS activity is detected in the same tissues, albeit at lower levels. These results suggest that the AS1 and AS2 promoters are both able to confer the same vascular-specific pattern of expression to GUS in older seedlings and in mature plants.

The AS1 promoter confers light-repressed expression to a GUS reporter gene

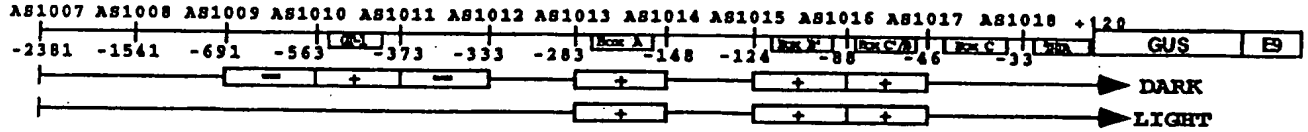
Previously, *in vitro* run-on assays in isolated nuclei showed that the transcription rate of the *AS1* gene is higher in dark-adapted plants compared to light-grown (Tsai and Coruzzi, 1991). Northern analysis showed that the steady state levels of AS1 mRNA in dark-grown or dark-adapted plants decreased 15-fold on transfer to the light (Tsai and Coruzzi, 1991). Here, we tested whether the AS1 promoter could confer negative light-regulation to a heterologous gene. As the AS1 and AS2 promoters showed similar patterns of GUS expression (see above), the promoter of the more highly expressed *AS1* gene was selected for deletion analysis. To define the cis-elements involved in light-repressed transcription, a series of 5′ deletions of the AS1 promoter were constructed, fused to the GUS reporter gene and transferred to tobacco plants (AS1007–AS1018) (see deletions, Figure 1c). Since the *AS1–GUS* gene was shown to be expressed only in the phloem cells of mature transgenic plants, an *AS1–GUS* construct was designed to optimize and to increase the stability and translatability of the GUS mRNA. All of the 5′ deletion constructs have the full 5′ untranslated leader of AS1 to +120, a modified GUS coding sequence from pRAJ275 (Jefferson *et al.*, 1986), and a 3′ non-coding region from the rbcs-E9 gene (see AS1007, Figure 1c) (see Experimental procedures for construction and sequence). The GUS sequence of pRAJ275 contains a consensus for eukaryotic translation flanking the ATG initiator codon of GUS (Clontech). These changes increased the average GUS activity of construct AS1007 (Figure 1c) by 10-fold compared to the non-optimized GUS contained in AS1001 (Figure 1b) (data not shown). The cell-specific pattern of expression of AS1–GUS was unchanged in the two constructs (AS1001 and AS1007). T_1 seeds of AS1 5′ deletion constructs AS1007–AS1018 were germinated on MSK media under continuous light or continuous dark for 10 days. For each construct, seedlings of at least 10 independent transformants were analysed histochemically for GUS expression. The shortest construct which retained expression was AS1016, which contained the –88 AS1–GUS construct. All of the transformants of AS1007 to AS1016 had the same pattern of expression. That is, GUS staining was detected only in the vascular tissue of the cotyledons, hypocotyl, and root tip in either light- or dark-grown 10-day old seedlings (see Figure 2a and b).

To measure differences in promoter strength of each deletion in light- or dark-grown seedlings, GUS activity was quantitated fluorimetrically (Jefferson, 1989). For all expressing AS1–GUS constructs (AS1007–AS1016), GUS activity was significantly higher in dark-grown seedlings (Figure 3b) compared to light-grown seedlings (Figure 3a). The median values (denoted by the bar) of dark-grown AS1007–AS1016 seedlings were consistently higher by about 2–4-fold compared to light-grown seedlings (Figure 3c). As controls, a promoterless construct (NN1019) and a constitutive promoter (PB41) were analysed (see Figure 1d). PB41 transgenic tobacco seedlings contain the construct 35S CaMV(4XB4 +A)–GUS, which has four copies of the 35S CaMV promoter subdomain B4 (–301 to –208) plus subdomain A (–90 to +8) fused to GUS (Benfey *et al.*, 1990). This PB41 construct was previously shown to have a GUS expression pattern in the vascular tissue of the hypocotyl and cotyledons of 10-day old seedlings (Benfey *et al.*, 1990). This cell pattern of PB41 is similar to the AS1–GUS containing seedlings described herein.

To determine whether the difference in the D:L ratios of various AS1 deletions was significant compared to the control (PB41) and compared to each other, a non-parametric unpaired test was performed. The *P* values between sets of constructs are summarized in Figure 3(e). All AS1 deletion constructs showed significant differences in D:L ratios compared to the control PB41 (*P* value <0.0001). Therefore, the AS1 promoter deleted down to 88 bps (AS1016) is able to confer light-repressed expression to the GUS reporter gene. The differences in D:L ratios between certain deletion constructs were also deemed significant by statistical analysis. For instance, significant increases or decreases in D:L ratio occurred between deletions AS1009–1010, AS1010–1011, AS1011–1012, and AS1013–1014 (Figures 3c and 3e). This suggests that these regions are involved in the light-regulated expression of the AS1 gene. The positive or negative effects of deleting *cis*-elements with regard to GUS expression in light- or dark-grown plants are summarized in Figure 3(d). Based on the median values, the GUS activity in dark-grown seedlings increases



(d)

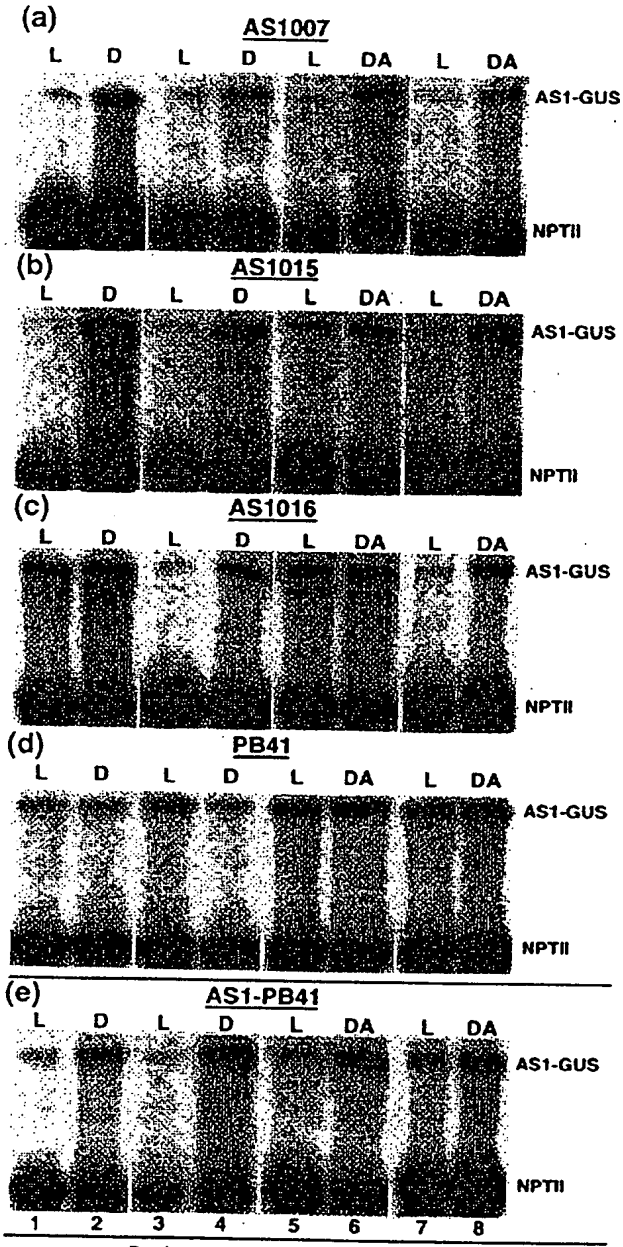


(e)

	PB41	AS1008	AS1009	AS1010	AS1011	AS1012	AS1013	AS1014	AS1015	AS1016
AS1007	<.0001*	.8722								
AS1008	<.0001*		.5185							
AS1009	<.0001*			<.0001*						
AS1010	<.0001*				<.0001*					
AS1011	<.0001*					<.0004*				
AS1012	<.0001*						.0985			
AS1013	<.0001*							.0103*		
AS1014	<.0001*								.2755	
AS1015	<.0001*									.3786
AS1016	<.0001*									
AS1-PB41	<.0001*									

*=Statistically significant (P<0.05)

Figure 3. Scatterplot of GUS activity from light and dark-grown AS1–GUS transgenic tobacco seedlings.
Extracts from 200–300 transgenic tobacco T_1 seedlings grown for 10 days in continuous light or dark on MSK media (100 ug ml^{-1} Kanamycin), were assayed for GUS activity.
Panel (a) GUS activity of transformants grown in the light.
Panel (b) GUS activity of transformants grown in the dark. Each dot represents the GUS activity of an independent transformant. GUS activity is expressed as nmoles of 4-methylumbelliferone (4-MU) produced per min per milligram protein. Bars indicate median values. *n*=number of independent transformants analysed.
Panel (c) D:L ratios of each deletion construct, where each dot represents the D:L ratio determined for each transformant and the median is indicated by the bar.
(d) A schematic representation (not drawn to scale) of the 5′ deletion endpoints of the AS1 promoter. Shown below is a diagram of the positive or negative effects on GUS expression in the dark and in the light after deleting certain *cis*-elements.
(e) A table of the non-parametric unpaired test on the D:L ratios between constructs. The D:L ratios of each construct (AS1007–AS1016) were compared to those of the control construct PB41 by a non-parametric unpaired test. The D:L ratios between constructs (ie., AS1007 versus AS1008, AS1008 versus AS1009, AS1009 versus AS1010, etc.) were also compared to determine if the differences in their D:L ratios are significant. The *P* values below 0.05 are considered significant. Non-parametric unpaired tests were performed with statistical program Instat™ 2.0.



(f)

	Dark vs Light D:L Ratio	2DA vs Light D:L Ratio
AS1007 (n=5)	1.9±.2 (1.6-2.1) P=.0043*	2.1±.3 (1.6-2.4) P=.0043*
AS1015 (n=8)	2.5±.5 (1.6-3.3) P=.0007*	1.9±.3 (1.5-2.1) P=.0007*
AS1016 (n=6)	1.8±.4 (1.3-2.3) P=.0043*	1.6±.2 (1.4-2.0) P=.0043*
PB41 (n=6)	1.2±.1 (1.0-1.3)	1.1±.1 (1.0-1.2)

(g)

AS1-PB41 (n=7)	3.1±.4 (2.0-3.5) P=.0012*	1.8±.2 (1.5-2.0) P=.0012*

*=Statistically significant (P<0.05)

when the region between –691 (AS1009) and –563 (AS1010), and –373 (AS1011) and –333(AS1012) are deleted (Figure 3b), with no significant change in expression in light-grown seedlings (Figure 3a). Therefore, this suggests that there was a negative element between those regions repressing AS1 expression in the dark (Figure 3d). By contrast, there appears to be a dark-specific enhancer between –563 (AS1010) and –373 (AS1011) (Figure 3b and d). Deleting the region between –563 and –373 decreased the median GUS activity of dark-grown seedlings with no significant change in light-grown seedlings (Figure 3a and b). Finally, the region between –283 (AS1013) and –148 (AS1014) appears to contain transcription elements that can effect levels of AS1 expression in light and dark-grown plants (Figure 3a and b).

For the remaining AS1–GUS deletion constructs, most deletions of the AS1 promoter did not significantly change the GUS expression levels or the D:L ratios. However, in one case, deleting the region –124 (AS1015) to –88 (AS1016) dramatically decreased the median GUS activity in both the light and dark-grown plants (Figure 3a and b) but did not change the median D:L ratio (Figure 3c). Here, the region from –124 to –88 appears to act as a positive element in the general transcriptional enhancement of the *AS1* gene (Figure 3d). Therefore, although the 88 bp AS1 promoter can maintain light/dark regulation, the 124 bp AS1 promoter (AS1015) is considered 'minimal' in that it confers light/dark expression of GUS activity at levels similar to those observed with larger fragments of the AS1 promoter (see Figure 3a and b).

The above results based on the GUS activity assays indicated that the AS1 promoter is able to confer light-repressed expression to a GUS reporter gene. To confirm that this represents transcriptional regulation, GUS mRNA

Figure 4. The AS1 promoter drives light-repressed expression of the GUS gene in transgenic plants.

RNase protection assays were performed with total RNA (30 μg) isolated from 300–400 transgenic tobacco plants grown in light (L), dark (D), or dark-adapted for 2 days (2DA). A 598 bp GUS and a 416 bp Kan riboprobe were used to detect the GUS and Kan mRNA. Shown in (a–d) are representative RNase protection assays of plants with 5′ AS1 deletion constructs; AS1007(–2381), AS1015(–124), AS1016(–88) and the control PB41(35S CaMV(4XB4+A)-GUS).

Shown in (e) are representative RNase protection assays of plants with the AS1 promoter from –124 to –33 fused to a heterologous promoter (35S CaMV(4XB4+A)-GUS), AS1–PB41. RNase protection assays were repeated, each independently, two times. The steady-state level of GUS and Kan mRNA were quantified by phosphor imaging. The D:L ratio of the transformants based on the steady-state level of GUS mRNA (relative to the Kan mRNA) in the light or dark/dark-adapted were determined.

Shown in (f) are the mean D:L ratios for AS1007, AS1015, AS1016 and the control PB41.

Shown in (g) is the mean D:L ratio for AS1–PB41. Represented are the mean D:L ratio ± SD with the range in parenthesis. *n*=number of independent transformants. The D:L ratios of each construct were compared to the control PB41 by a non-parametric unpaired test. *P* values below 0.05 are considered significant.

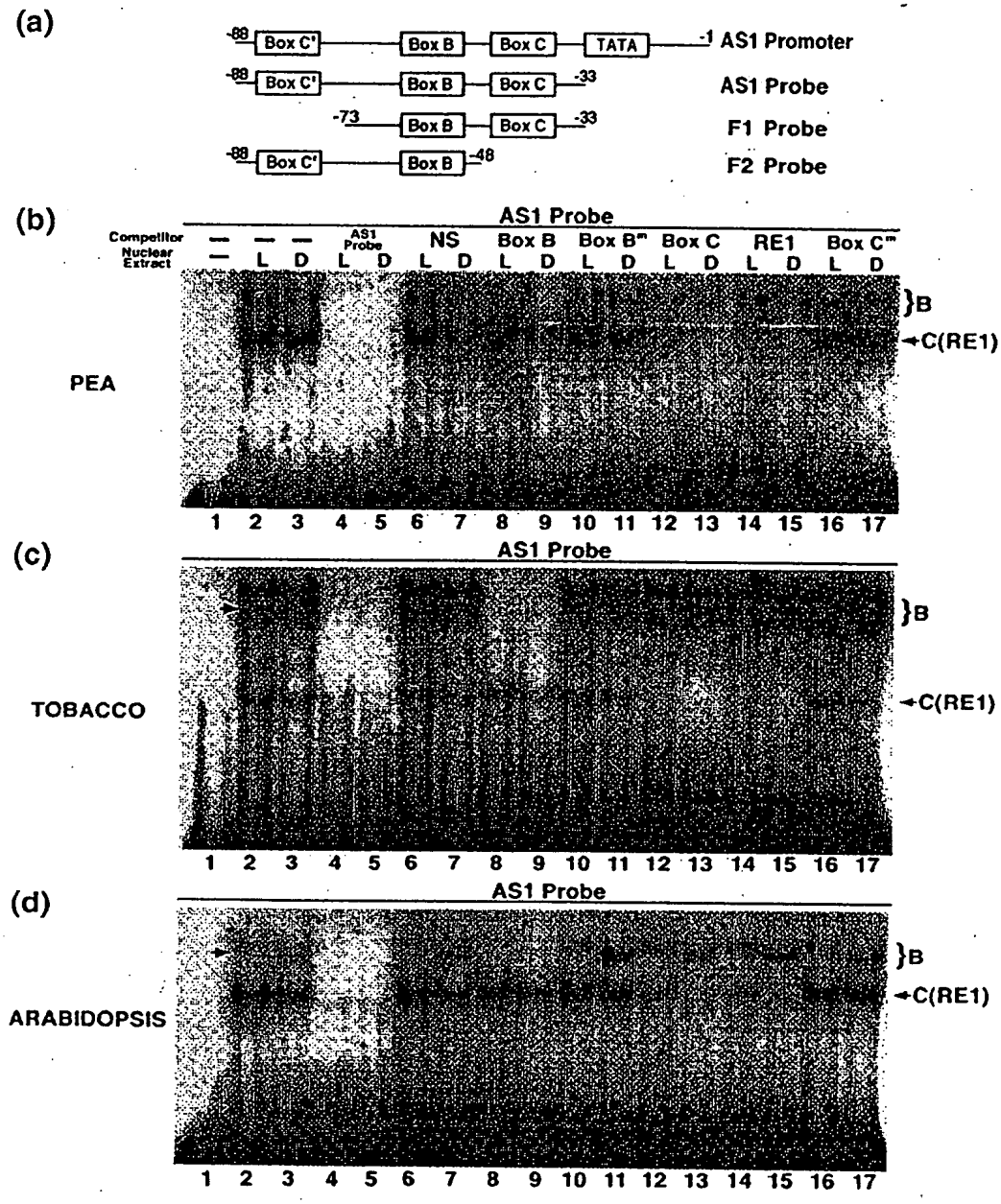
levels were analysed from all GUS expressing AS1–GUS transgenic plants (AS1007 to AS1016). GUS mRNA levels are shown for representative plants AS1007 (–2381 AS1–GUS), AS1015 (–124 AS1–GUS), and AS1016 (–88 AS1–GUS) (Figure 4a–c). The quantification of RNA in (5–8) independent transformants are tabulated in Figure 4(f). RNA was extracted from T_1 seedlings germinated and grown for 10 days in continuous light (*L*) versus continuous dark (*D*), or plants that were light-grown (*L*) (18 days) and subsequently dark-adapted (*DA*) (2 days) (see Experimental procedures). A large number of seeds for each transformant were used (300 seeds per transformant) to reduce the variability of a heterogeneous population derived from the primary transformants. RNA from seedlings of 5 to 8 individual transformants of AS1007, AS1015, and AS1016 were analysed by RNase protection assay. Figure 4(a–c) shows representative RNase protection assays of constructs AS1007, AS1015, and AS1016, two independent transformants for each. In each AS1–GUS construct, the steady-state level of the GUS mRNA was significantly and consistently higher in seedlings grown in the dark or dark-adapted compared to those grown in continuous light (Figure 4a–c). As an internal control, the mRNA for the constitutively expressed nptII mRNA was also examined (Odell *et al.*, 1985). The level of GUS mRNA and nptII mRNA were quantified by phosphor imaging (Figure 4f). GUS mRNA measured in light-grown and in dark-grown or dark-adapted plants was normalized to the level of nptII mRNA. Shown in Figure 4(f) are the average D:L ratios of GUS mRNA for the independent transformants AS1007, AS1015, and AS1016. The steady state level of GUS mRNA in dark-grown or dark-adapted AS1–GUS seedlings was approximately 2- to 3-fold higher than the level of GUS mRNA in light-grown seedlings. As a control, 35S CaMV (4XB4 +A)–GUS containing seedlings (PB41) showed no difference in the levels of GUS mRNA in response to the light or dark treatment (representatives shown in Figure 4d) with an average D:L ratio of 1.2 (for dark- versus light-grown) or 1.1 (for dark-adapted versus light-grown) (Figure 4f). All AS1–GUS constructs were shown to have a significant increase in the level of dark expression compared to PB41 (*P* values 0.0007–0.0043, Figure 4f). Thus the AS1 promoter was able to confer negative regulation by light to the GUS reporter gene in transgenic tobacco plants.

124 bp AS1 element confers light-repression to a heterologous promoter

By 5′ deletion analysis, it was shown that 124 bp of the AS1 promoter can confer light-regulated expression to a reporter gene roughly equivalent to the quantity and quality of expression conferred by the longest AS1–GUS construct. To determine if the 124 bp sequence could confer light-regulated expression to a heterologous promoter, the AS1 promoter from –124 to –33 was placed upstream to a 35S CaMV(4XB4+A)–GUS element (PB41) (Figure 1e). Numbers are relative to the start of transcription (+1). The AS1–PB41 construct was introduced into tobacco and the subsequent transgenic tobacco plants were analysed for GUS activity. Approximately 200–300 T_1 seedlings were grown on MSK media for 10 days in continuous light or continuous dark. For each independent transformant analysed, the GUS activity was consistently higher in the dark-grown seedlings (Figure 3b) compared to those grown in the light (Figure 3a). The D:L ratio of 26 independent AS1–PB41 transformants is shown as a scatterplot in Figure 3(c) with a median D:L ratio of 2.5. A non-parametric unpaired test between the D:L ratios of AS1–PB41 and the control PB41 showed their differences to be significant with a *P* value <0.0001 (Figure 3e). A significant difference between the GUS activities of the light-grown AS1–PB41 versus PB41 was also observed. In light-grown seedlings, the median GUS activity of AS1–PB41 (0.519 nmol 4 MU min^{-1} mg^{-1}) was significantly lower than that of PB41 (0.794 nmol 4 MU min^{-1} mg^{-1}) with a *P* value of 0.0383. In dark-grown seedlings, the median GUS activity for AS1–PB41 (1106 nmol 4 MU min^{-1} mg^{-1}) was not (significantly) different to those of PB41 (1077 nmol 4 MU min^{-1} mg^{-1}) with a *P* value of 0.5398. This data suggests that the 124 bp AS1 promoter fragment is specifically repressing transcription of PB41 in the light. To determine if the 124 bp AS1 promoter confers light-repression to a heterologous promoter at the RNA level, GUS mRNA from AS1–PB41 transgenic plants was analysed by RNase protection assay. RNA was extracted from T_1 seedlings (300 to 400 seedlings) of seven individual AS1–PB41 transformants grown on MSK medium for 10 days under continuous light (L) versus continuous dark (D), or from plants grown 18 days in continuous light (L) and then dark-adapted (DA) for 2 days. Representative RNase protection assays of two independent transformant are shown in Figure 4(e). Quantitation of the GUS mRNA level relative to the level of nptII mRNA by phosphor imaging showed a 2- to 3-fold increase in the GUS mRNA in dark-grown or dark-adapted AS1–PB41 compared to those grown in the light (Figure 4g). Thus, the 124bp AS1 promoter was able to confer light-induced repression to a heterologous promoter.

In vitro *gel-shift analysis of the AS1 promoter identifies putative repressor binding sites, Box B and Box C/C′*

The AS1 5′ deletion analysis showed that plants containing only 88 bp of the AS1 promoter can confer light-repressed expression (to GUS) (Figure 5). Therefore, the 88 bp sequence of the AS1 promoter contains cis-elements that mediate the light-repression of the AS1 gene. To explore which DNA sequences contained within these 88 bp of AS1 might be involved in the light-repressed regulation, the



AS1 promoter sequence was compared to the promoters of two other pea genes that are transcriptionally repressed by light; pea *AS2* (Tsai and Coruzzi, 1990) and *pea phytochrome* (Sato, 1988). Two short sequences downstream of –88 in pea *AS1* have been identified as candidates for negative light-regulatory elements based on DNA homology to *AS2* and *pea phytochrome*; Boxes B and C (see Figure 1a for sequence). In addition, the Box C sequence has homology to an inverse sequence of RE1, a sequence shown to act as a repressor in the oat phytochrome gene which is also negatively regulated by light (Bruce *et al.*, 1991) (For sequence, see Figure 5 legend). To identify whether these conserved DNA sequences are involved in DNA–protein interactions, *in vitro* gel-shift analysis was performed. In the experiments shown in Figure 5(b–d), the 88 bp AS1 promoter DNA fragment (–88 to –33) was used as a labelled DNA probe (AS1 probe). Nuclear extracts tested were from leaves of peas grown in the light (PL) or dark (PD) (Figure 5b), from tobacco grown in the light (TL) or dark-adapted for 4 days (TD) (Figure 5c), or with nuclear extracts from leaves of *Arabidopsis* grown in the light (AL) or dark-adapted for 6 days (AD) (Figure 5d). Following

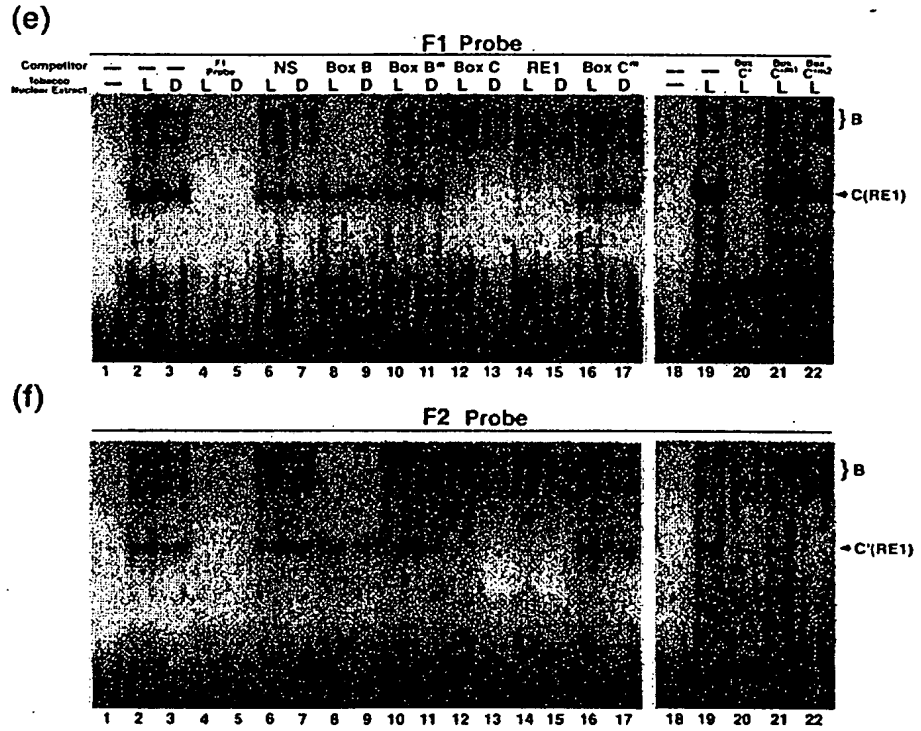


Figure 5. DNA:protein complexes in minimal functional AS1 promoter element.
(a) A schematic presentation of the DNA fragments of the AS1 promoter used as a probe to detect DNA-protein bindings by gel-shift analysis.
(b–d) Gel-shift analysis using the AS1 probe (–88 to –33) to detect DNA protein bindings. The AS1 probe has Box B, Box C, and Box C' (see Figure 1a for sequence).
(e–f) Gel-shift analysis using F1 (–73 to –33) and F2 (–88 to –48) as a probe. F1 has Box B and Box C. F2 has Box B and Box C'. For competition assays, DNA fragments were generated by annealing two complimentary oligos. Competitors are Box B=AAACGACACCGTTT, Box B^m=AAACGAAAAAGTTT, Box C=AGCTCCCACCTTC, Box C^m=AGCTCAAACCTTC, Box C'=TCCCGGTACACACTTCTT, Box C'^{m1}=TCCCGGTAAAAACTTCTT, Box C'^{m2}=TCAAGGTACACACTTCTT, and a dimer of RE1=CCGCGCCCATG (Bruce *et al.*, 1991) (Bold letters identify mutated residues (C→A) in mutants). NS (non-specific DNA fragment) is a gel purified 63 bp XbaI/*Sal*I DNA fragment of Bluescript generated by PCR. Gel-shift analysis was performed with nuclear extracts from leaves of pea grown in the light (L) or dark (D), from tobacco grown in the light (L) or dark-adapted for 4 days (D), and from *Arabidopsis* grown in the light (L) or dark-adapted for 6 days (D).

electrophoresis, shifts were observed with all nuclear extracts indicating the formation of DNA–protein complexes (Figure 5b–d, lanes 2 and 3). The complexes detected in PL and PD were identical in mobility (Figure 5b). The complexes detected in TL and TD were also very similar except for an extra band detected in TL (indicated by an arrow, Figure 5c, lane 2). In *Arabidopsis*, the complex forming the upper band is distinct in mobility in extracts from light-grown (AL) versus dark-adapted (AD) plants (indicated by an arrow, Figure 5d, lane 2). The protein factors involved in these shifts were shown to be specific for the AS1 promoter and conserved between species as determined by competition experiments. The shifts are each competed with a 50-fold excess of unlabeled 88 bp AS1 promoter DNA fragment (Figure 5b–d, lanes 4 and 5), but not by a 50-fold excess of a non-specific (NS) DNA fragment of similar size (Figure 5b–d, lanes 6 and 7). To localize the specific DNA binding sequences within the –88 to –33 bp AS1 fragment, competition experiments were performed. The upper shifted bands detected with pea, tobacco, and *Arabidopsis* extracts were confirmed to be the result of a protein interaction with DNA element Box B. The Box B shift is competed by an excess of unlabeled wild-type Box B (Figure 5b–d, lanes 8 and 9), but not by an excess of unlabeled mutant Box B^m (Figure 5b–d, lanes 10 and 11) (for sequence, see Figure 5 legend.) The lower band detected is defined as a Box C:protein interaction as it can be competed by an excess of unlabeled Box C (Figure 5b–d, lanes 12 and 13) but not by an excess of mutant Box C^m (Figure 5b–d, lanes 16 and 17) (see Figure 5 legend for sequences). The DNA–protein binding of both Box B and C are each eliminated when the cytosine nucleotides in each are changed to adenine (see Figure 5, legend). These cytosine nucleotides are therefore deemed to be required for DNA–protein binding. The Box C shift is also specifically competed by RE1, a putative repressor element previously defined in the oat phytochrome gene (Bruce *et al.*, 1991) (Figure 5b–d, lanes 14 and 15). We note that either Box C or RE1 elements do not compete as well (for the Box C shift) in extracts of light-grown plants (Figure 5b–d, lanes

12 and 14) compared to extracts of dark-grown/adapted plants (Figure 5b–d, lanes 13 and 15). This difference could either reflect enhanced binding activity in extracts of light-grown plants or it could be an artifact of extract preparation.

Further *in vitro* gel-shift analysis using the smaller F1 and F2 probes revealed that there are two *cis*-elements (Box C and Box C') that can be competed by the RE1 element (Bruce *et al.*, 1991) within the 88 bp AS1 fragment (Figure 5a). F1 encompasses the AS1 promoter from –73 to –33, while F2 encompasses the AS1 promoter from –88 to –48 (Figure 5a). Both F1 and F2 contain the Box B element. F1 contains a Box C element while F2 contains a Box C' element (see Box C', Figure 1a for sequence). The F1 and F2 probes were used to detect complexes formed with tobacco, pea, and *Arabidopsis* extracts. While all gave similar results, only the tobacco extracts are shown (Figure 5e and f), as the *in vivo* promoter dissections were performed in tobacco. Incubation with F1 and F2 probes showed similar shifts (Figure 5e and f). As determined by competition experiments, these shifts were found to be specific for the F1 and F2 probes. The shifts are competed with a 50-fold excess of unlabeled F1 or F2 DNA fragments (Figure 5e and f, lanes 4 and 5), but not by a 50-fold excess of non-specific DNA fragment (Figure 5e and f, lanes 6 and 7). Both F1 and F2 have a DNA:protein interaction with DNA element Box B (upper band, Figure 5e and f). Competition experiments showed that the upper shifted band can be competed by an excess of unlabeled wild-type Box B (Figure 5e and f, lanes 8 and 9), but not by an excess of unlabeled mutant Box B^m (Figure 5e and f, lanes 10 and 11; see Figure 5 legend for sequences). The lower band was found to be a DNA:protein interaction with Box C or Box C'. Using F1 or F2 as a probe, the lower band can be competed by an excess of unlabeled Box C (Figure 5e and f, lanes 12 and 13), but not by an excess of mutant Box C^m (Figure 5e and f, lanes 16 and 17; see Figure 5 legend for sequences). Similarly, the lower band can also be competed by Box C' (Figure 5e and f, lane 20) and not by an excess of mutant Box C'^{m1} (Figure 5e and f, lane 21). Competition with another Box C' mutant, Box C'^{m2} (see Figure 5 legend for sequence), can compete out the lower band detected with the F2 probe but not with the F1 probe (Figure 5e and f, lane 22). The cytosine nucleotides that have been changed to adenine in Box C'^{m2} (see Figure 5, legend) seemed to be involved in the DNA:protein binding of Box C but not in Box C'. The Box C or C' shift can each be competed by the RE1 element (Figure 5e and f, lanes 14 and 15). Competition experiments using pea and *Arabidopsis* nuclear extracts also showed a Box C' shift which can be competed by the Box C and RE1 elements (not shown). The sequences shared between Box C, C', and an inverted sequence of RE1 are shown in Figure 6. The nuclear protein factor that binds to either Box C, C', or RE1 could be a repressor binding protein which has multiple DNA binding sites. Alternatively, there could be a family of binding proteins that bind to Box C, C', or RE1 which may be involved in light-activated transcriptional repression.

Discussion

The expression of the *AS1* and *AS2* genes of pea has been shown to be regulated by light in a negative manner (Tsai and Coruzzi, 1990). For *AS1*, it was shown that at least part of this negative light-regulation occurs via the phytochrome photoreceptor (Tsai and Coruzzi, 1990). The light-repressed expression of *AS* genes is also evident in other species such as tobacco and *Arabidopsis* (Lam *et al.*, 1994; Tsai and Coruzzi, 1991). Here, we analysed the pea AS1 promoter in transgenic tobacco to define the *cis*-acting DNA elements and the protein factors that bind to these elements involved in light-activated transcriptional repression.

The sequence of the AS1 and AS2 genomic fragments containing the 5' upstream region and part of the transcribed region are reported here. The putative TATA boxes are perfectly conserved between the *AS1* and *AS2* genes at –30. The overall sequence homology between 5' upstream regions of the *AS1* and *AS2* genes from nucleotide –600 to –1 is less than 50%. However, some highly conserved sequences were found regionally within the TATA proximal 200 bases of the AS1 and AS2 promoters (Figure 1a and 1f). Histochemical analysis of GUS activity in transgenic tobacco plants carrying either AS1–GUS or AS2–GUS promoter fusion constructs reveals that both AS1 and AS2 promoters confer the same organ- and cell-specific expression patterns to the GUS reporter gene. However, the AS1 promoter was more robust than AS2 in all transformants examined. This difference is also reflected in the level of AS1 and AS2 mRNA in pea (Tsai and Coruzzi, 1990). These results suggest that although the AS1 and AS2 promoters both contain similar cis-elements for tissue-specific and cell-specific expression, the AS1 promoter most likely contains additional enhancer elements.

Both AS1 and AS2 promoters are able to direct GUS expression in the vascular tissue, primarily in the phloem of cotyledons, leaves, stems, and roots. These cell-specific expression patterns conferred by the AS promoters provide insight into the mechanism of asparagine synthetase in plants. Physiological studies have shown that asparagine is transported in the phloem and that this transport follows a 'source-to-sink' rule (Sieciechowicz *et al.*, 1988). Asparagine synthesized at sources is loaded into vascular elements and transported to the various sinks such as growing shoots, roots, developing fruits and seeds (Dilworth and Dure, 1978). The AS–GUS studies reported here demonstrate that at least some of the asparagine transported in the plant vasculature is synthesized in the phloem cells *in situ*. It is curious that neither the AS1 nor AS2 promoters are expressed in mesophyll cells of mature leaves where

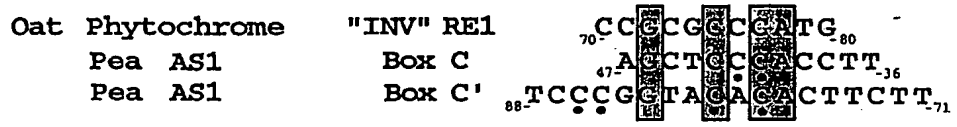


Figure 6. Sequence comparison between RE1, Box C, and Box C'.
Identical nucleotides between an inverted sequence of the RE1 element from the oat phytochrome gene (Bruce *et al.*, 1991) and Box C and C' from the pea *AS1* gene are shaded. Cytosine nucleotides changed to adenine to create (mutants) Box C^m, Box C'^{m1}, and Box C'^{m2} are dotted.

asparagine is thought to play a minor role in photorespiration (Lea and Miflin, 1980). While these are the only two glutamine-dependent *AS* genes of pea defined by Southern hybridization and cDNA analysis (Tsai and Coruzzi, 1990), the possiblity remains that peas contain other less homologous *AS* genes that are expressed in mesophyll cells.

The AS1 promoter was chosen for *cis*-element analysis owing to its increased promoter strength relative to the AS2 promoter. In analysing a series of AS1 5′ promoter deletion mutants, the shortest promoter fragment tested which was able to confer light-regulated expression to GUS was –88 bp (AS1016). Plants with the 'full-length' –2381 bp AS1 promoter (AS1007), and all deletions down to –88 bp (AS1016) had an overall GUS activity which was higher in the dark by 2- to 4-fold compared to those grown in the light. The –124 promoter is designated as minimal as it was able to confer the identical level of expression to GUS as the 'full-length' promoter. A high basal level of AS1–GUS expression in light-grown seedlings was observed. This high level of AS1 expression most likely reflects a basal level owing to a light-independent function of AS1 which was previously reported for pea cotyledons (Tsai and Coruzzi, 1990). Deleting certain *cis*-elements of the AS1 promoter affected the median D:L ratios, suggesting their involvement in the light-regulation of AS1 (Figure 3d). Deleting the two GT-1 DNA binding sites (Tsai and Coruzzi, unpublished data) located at nucleotide –444 to –422 (see Figure 1a and c) (between deletions AS1010 and AS1011) results in a dramatic reduction in the level of dark expression with no significant effect on light expression (Figure 3d). Thus, the GT-1 binding sites appear to be involved in the specific expression of the *AS1* gene in dark-grown plants. GT-1 binding sites have been found in the promoters of other light-regulated genes including pea rbcS-3A (Green *et al.*, 1987, 1988) as well as in the light-induced nuclear gene encoding GS2 (the chloroplast form of glutamine synthetase) in pea (Tjaden *et al.*, 1995) and the light-repressed gene for phytochrome in rice (Kay *et al.*, 1989) and oat (Bruce *et al.*, 1991). Deleting the Box A and B′ elements conserved between AS1, AS2, and *pea phytochrome* affected levels of both light and dark (GUS) expression (Figure 3d). This suggests that Box A and Box B′ may be transcriptional elements involved in basal expression. The 5′ deletion of Box B and Box C/C′ elements conserved between AS1, AS2, and pea phytochrome destroyed transcription (AS1017 and AS1018) (Figure 3a and b).

In a gain-of-function experiment, the AS1 promoter fragment from –124 to –33 was shown to confer light-induced repression to a constitutive 35S CaMV promoter, 35S CaMV (PB41). The (median) GUS activity of light-grown AS1–PB41 was significantly lower than those of the controls (PB41), while dark expression of AS1–PB41 was unaffected (see Results and Figure 3a and b). This suggests that the light-regulation of AS1 transcription involves light-activated repression and not dark-activation, although the latter can not be completely ruled out.

Gel shift analysis showed two major DNA/protein complexes involving the Box B and Box C/C′ elements contained within the shortest light-repressed –88 promoter (AS1016). The B and C/C′ binding factors are present in extracts of both light or dark-grown/adapted plants. However, in light-growth conditions, the protein(s) binding to Box B are modified. That is, there is an extra 'B′' shift observed in tobacco (see arrow, Figure 5c, lane 2) and the B complex is altered in mobility in light-grown compared to dark-adapted *Arabidopsis* (see arrow, Figure 5d, lane 2). This light-specific modification of the 'B′' shift suggests that there is a light-induced modification of the B-binding protein or a light-induced modification of a protein that binds to the B-binding protein. This light-induced modification of the B-binding protein may be involved in light-activated transcriptional repression of AS1. In addition, the nuclear protein that specifically binds to Box C or Box C′ may also be involved in repression, as its binding is competed by RE1, a putative repressor element previously defined in the oat phytochrome gene (Bruce *et al.*, 1991). The Box C element is immediately 5′ proximal to the TATA box, which is a typical placement for a classical repressor that interferes with RNA polymerase binding in bacteria (McClure, 1985). Further experiments are necessary to answer the question as to how B and C/C′ DNA–protein complexes might be involved in light-activated transcriptional repression. The two Box C elements (C and C′) can compete with each other for factor binding. The nuclear protein that binds to the C or C′ elements may be the same or it could be different. While one mutant of Box C′ (Box C'^{m1}) affects binding to both Box C and C′, the cytosine nucleotides mutated in Box C'^{m2} only affected factor binding with Box C and not with Box C′. These results suggest that there may be a preference of binding of the factor(s) to either Box C or Box C′ site. As C and C′ binding are each competed with the putative repressor element RE1 defined in the phytochrome (Bruce *et al.*, 1991), they along

with the B element may act as repressor-binding DNA elements in the AS1 promoter. In *Arabidopsis*, the *ASN1* gene has been shown to be repressed by either light or sucrose (Lam *et al.*, 1994). Likewise, the pea AS1 promoter activity appears to also be repressed by sucrose (data not shown, Ngai and Coruzzi, unpublished). Thus, it would be interesting to determine whether light-repression and sucrose-repression of pea AS1 are mediated by the same or distinct trans-acting factors or *cis*-elements. The identity of 'repressor' elements in AS1 would be useful for engineering a promoter for temporal expression of foreign genes in plants in which foreign gene expression could be repressed by light and/or sucrose.

Experimental procedures

Isolation of AS genomic clones from peas

AS1 and AS2 genomic clones were isolated from *Pisum sativum* (var. 'Feltham First') genomic library constructed in λEMBL3 (Lycett *et al.*, 1985). This genomic library was probed with 1.4 kb *Sst*I/*Bam*HI fragment of cDNA clone cAS1 (Tsai and Coruzzi, 1990). Two clones were isolated, λgAS1 which hybridized strongly to the AS1 cDNA probe, and λgAS2 which hybridized more weakly to the AS1 cDNA probe. The inserts of λgAS1 and λgAS2 are 17 kb (gAS1) and 14 kB (gAS2) respectively. A 3.2 kb *Sst*I/*Bam*HI fragment of λgAS1 and a 2.2 kb *Bgl*II fragment of λgAS2 were subcloned into pTZ18U vectors to create the plasmids gAS1.a and gAS2.b, respectively. The 5' upstream region of *AS1* and *AS2* genes was determined by Southern hybridization using the 5' end of AS1 and AS2 cDNAs as probes (data not shown). The 5' upstream region of *AS1* and *AS2* gene are located within gAS1.a and gAS2.b (Figure 1).

RNase protection assay

The GUS and nptII mRNAs were detected by RNase protection assay. Plasmid pGEM32 subclones carrying either kanamycin (352bp) or GUS (534bp) cDNA fragments were linearized by digestion with *Hin*cII and *Hin*dIII, respectively, and subsequently used to make P^{32}-CTP labeled nptII and GUS antisense riboprobes by *in vitro* transcription using SP6 RNA polymerase (Melton, 1984). Total RNA used in the assays were extracted from plant tissue using a buffer as described by Jackson and Larkins (1976). Total RNA was treated with RQ1 DNase (Promega) to remove any DNA contamination. The detection of GUS and nptII mRNA was performed using an RPAII™ribonuclease protection assay kit (Ambion). Briefly, 30 μg of total RNA isolated from transgenic tobacco seedlings was hybridized with an excess of radiolabeled GUS and kanamycin riboprobes overnight in 80% deionized formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4 1 mM EDTA at 42°C. After hybridization, single-stranded probes were degraded with a mixture of RNase A and RNase T1 enzymes. Digested products were separated on a 6% acrylamide, 7 M urea gel, and exposed to X-ray film at –80°C. The steady-state level of the GUS and nptII mRNA of each transformant was quantified by phosphor imaging (Model GS-250 Phosphor Imaging System from Biorad). The GUS mRNA measured in the light and in the dark or dark-adapted were normalized to the level of nptII mRNA.

Construction of promoter-GUS plasmids

The AS1–GUS construct, AS1001 was created by subcloning a *Bam*HI/*Pvu*II fragment (nt –2381 to +10) from gAS1.a to a *Bam*HI/*Pvu*II site of pBI101.1 vector (Jefferson, 1989). For the AS2–GUS construct, a *Bgl*II/*Bgl*II fragment (nt –998 to +1240) from gAS2.b were subcloned into pBI101.1 to create AS2002. A series of 5' AS1 promoter deletions (AS1007–AS1018) were constructed by fusing different 5' ends of the AS1 promoter to GUS. For constructs AS1010–AS1018, each of the different 5' AS1 deletion fragments (–n to +120) were generated by PCR using specific oligonucleotides. The PCR fragments were subcloned as an *Hin*dIII/*Nco*I fragment into the pTZ-GUS plasmid. The pTZ-GUS plasmid contains a GUS coding sequence from the plasmid pRAJ275 (Clontech), which has the eukaryotic consensus sequence for translation flanking the initiator codon (CATACCATGGTCCGT) and a 3' end from the *rbcs-E9* gene (Coruzzi *et al.*, 1984). After verifying the promoter sequences generated by PCR to be correct by DNA sequence analysis, the different 5' AS1–GUS fragments from pTZ–GUS were subcloned into pBI101.1 as a *Hin*dIII/*Eco*RI fragment to create AS1010–AS1018. A *Sal*I/*Sca*I fragment from AS1001 (nt –2381 to –1) was subcloned into AS1010 to create AS1007. A *Hin*dIII fragment from AS1001 (nt –1541 to –596) was subcloned into AS1007 to create AS1008. A *Cla*I/*Sna*BI fragment from AS1007 was subcloned into BS–GUS, a plasmid similar to PTZ–GUS. This created a *Sal*I/ScaI fragment (nt –691 to –1) which was then inserted into AS1010 to create AS1009. NN1019 is a promoterless-GUS construct. The control consruct, PB41 has four copies of the subdomain B4 (–301 to –208 of the 35S CaMV promoter) plus A (–90 to +8) fused to GUS and was generated as described in Benfey *et al.* (1990). The gain-of-function construct, AS1–PB41 was created by inserting a PCR generated AS1 promoter from nt –124 to –33 into a *Hin*dIII site just upstream to the PB41 element. All constructs were checked by restriction digest and DNA sequencing. Constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating as described by Bevan (1984) or by electroporation (Gene Pulser™ by Biorad).

Growth conditions of transgenic tobacco plants

Nicotiana tabacum cv. SR1 was transformed using the leaf disc method (Horsch *et al.*, 1985). The Kan^R primary transgenic tobacco plants were grown in sterile culture, then transferred to soil and grown in a normal day/night cycle (16 h of light at 26°C and 8 h of dark at 22°C) for maturation. T1 seeds collected were sterilized in 10% sodium hypochloride and grown vertically on square plates (100×15 mm) on MS media without sucrose and 100 μg ml^{-1} kanamycin. Plates were incubated at 4°C for 2 days, then transferred to white-light (22°C) or wrapped in aluminum foil and kept in a dark chamber (22°C) for 10 days; or grown in continuous white-light for 18 days and dark-adapted for 2 days. Three hundred to 400 seedlings were harvested for RNA extraction. Harvesting of dark-grown seedlings was performed under a green safe-light (Kodak 7B safe-light filter, cat#8483141). Histochemical assays of GUS enzyme was performed on transgenic tobacco seedlings as described in Benfey *et al.* (1989).

Photomicrographs were taken with a Nikon Optiphot microscope. The GUS activity of transgenic tobacco seedlings (200–300) was measured fluorometrically as described by Jefferson *et al.* (1989). Protein concentrations were determined using the Bio-Rad protein assay with BSA as a standard (Bradford, 1976).

Gel-shift analysis

Gel-shift analysis was performed to identify DNA–protein interactions within the 88 bp AS1 promoter. A DNA fragment of the

AS1 promoter from –88 to –33 was generated by PCR, digested with restriction enzymes, and gel purified. These were then labeled using the Klenow enzyme and (^{32}P)dCTP and (^{32}P)dATP (NEN). Nuclear extracts were prepared by the method of Green *et al.* (1987,1989) from leaves of light or dark-grown pea, from leaves of tobacco plants either grown in the light or dark-adapted for 4 days, or from *Arabidopsis* (ecotype, Columbia) plants either grown in the light or dark-adapted for 6 days. The assays were performed by incubating 1–5 ng labeled restriction fragments (20–30 000 cpm μl^{-1}) with nuclear extracts from pea, tobacco, and *Arabidopsis* in a 10 µl reaction solution containing 4.0 µg of poly(dI-dC) (Pharmacia) and 1 mM EDTA in NEB buffer (25 mM Hepes-KOH, pH 7.5, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1.0 mM DTT, and 5 mM $MgCl_2$) for 15 min at room temperature. For competition studies, 50-fold excess of unlabeled probe or specific DNA competitors was added before adding labeled DNA fragment. Specific DNA competitors were generated by annnealing two complimentary oligos. The competitors used in the assay are Box B=AAACGACACCGTTT, Box B^m=AAACGAAAAAGTTT, Box C=AGCTCCCACCTTC, Box C^m=AGCTCAAACCTTC, and RE1=CCGCGCCCATGCCGCGCCCATG (a dimer of the RE1 element CCGCGCCCATG). The competitor NS (non-specific) is a gel purified 63 bp XbaI/*Sal*I DNA fragment of Bluescript generated by PCR. Following incubation, the mixture was electrophoresed on a non-denaturing 6% acrylamide gel in 0.5 × TBE, gels were dried onto DE81 paper, and autoradiographed at –80°C.

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